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THE UTERUS

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THE UTERUS*

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JOSEPH T. VELARDO

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INTRODUCTION

By Joseph T. Velardo

Department of Anatomy, Yale University School of Medicine, New Haven, Conn.

Numerous fields of specialization within the biological sciences are contributing information to the rapidly advancing area of knowledge concerned with the endocrincology of reproduction. Before endocrinology was recognized as a field of study in its own right, the early 1920s found anatomists, embryologists, physiologists, and zoologists investigating what was to be a most highly specialized area of veterinary and human medicine. Today, biochemists (those concerned with organic molecules as well as those involved with enzymatic analyses), demographers, geneticists, gynecologists, obstetricians, pathologists, and internists have joined ranks with the basic scientists to assist in the investigative work in problems concerned with reproductive mechanisms. As a matter of fact, the living history of the endocrinology of reproduction, with all of its animated backbones, received the greatest impetus from anatomists and zoologists. Early researches that have contributed to a significant and rapidly expanding accumulation of knowledge regarding the endocrinology of reproduction include the investigations of Edgar Allen on the estrous cycle of the mouse; F. L. Hisaw's study of the estrous cycle of the rat and his researches on the physiology of estrogen, progesterone, and relaxin; H. M. Evans and J. A. Long's description of the estrous cycle of the rat; Evans and Li's work on the pituitary hormones; P. E. Smith's discovery and perfection of a technique for hypophysectomy and the observations of its effects; G. B. Wislocki's school of histochemistry and comparative placentology; George W. Corner and W. M. Allen's research on the isolation of crude progesterone; Elizabeth M. Ramsey's studies on implantation; S. R. M. Reynold's numerous studies on pregnancy and parturition; Louis B. Flexner's work on fetal growth and nutrition; F. R. Lillie's research on chemistry and fertilization; Carl R. Moore's work on the biology of the testes; Edgar Allen, G. W. Bartelmez, C. G. Hartman, J. E. Markee, and F. L. Hisaw's researches on menstruation; and R. O. Greep and H. B. Van Dyke's investigations on the physiology of the gonadotropins.

It has been almost twenty years since a definitive statement has been made concerning the physiology of the uterus. Furthermore, endocrinology is so complex that it is almost impossible for one or a few individuals to summarize the available knowledge, perspectives, and perplexing questions that exist in the area of uterine physiology. Therefore, a monograph on the uterus bringing together the work of numerous specialists in this area of the physiology of reproduction is not only timely, but most important for an evaluation of our present concepts.

During the past two decades a great deal of work has been done in a variety of bisciplines by a large number of investigators dealing, in general, with problems related to human reproduction and the female reproductive tract. While it would not be possible to cover thoroughly the entire field in one publication, the work of a carefully selected group of investigators from a variety of disciplines—anatomists, physiologists, endocrinologists, biochemists, pharmacologists, pathologists, obstetricians, gynecologists, and clinical investigators—has been included here in an effort to summarize the work of the past two decades and to correlate the work done in any one field with that done in others. Particular emphasis is given to problems that remain unsolved and even undefined in order to indicate effective leads for further investigation.

I introduce the subject matter of this publication by re-echoing a thought of Lord John Wilmot, Earl of Rochester. Although much has been and will be said of the uterus, it was Lord John who aptly summarized the importance of this organ when he said: "On this soft anvil all mankind is made."

It is a sincere pleasure to record here the kindly personal assistance so unselfishly given to me by my two able co-chairmen: William B. Ober, of Knickerbocker Hospital, the College of Physicians and Surgeons of Columbia University and the Albert Einstein College of Medicine, New York, N. Y.; and Herbert G. Winston, of the Albert Einstein College of Medicine, of the New York Medical College, and of the Metropolitan Hospital, New York, N. Y. We are especially thankful to Carl G. Hartman, Ortho Research Foundation, Raritan, N. J.; William U. Gardner, Yale University School of Medicine, New Haven, Conn.; Roy O. Greep, Harvard Medical School, Boston, Mass.; and George W. Corner of The Rockefeller Institute for Medical Research, New York, N. Y., for their numerous helpful suggestions and friendly counsel.

Part I. Historical and Morphogenetic Considerations

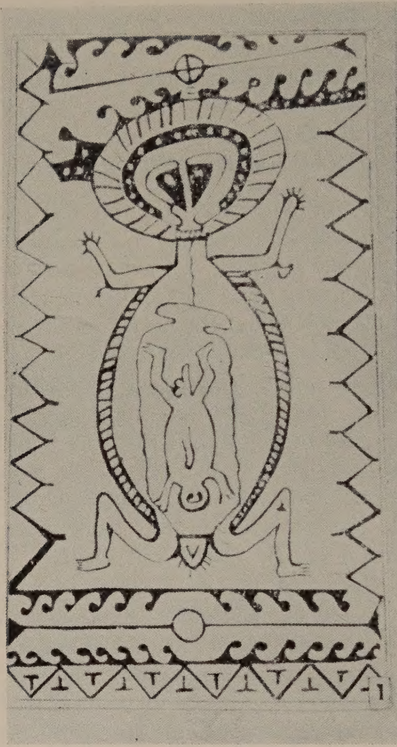
HISTORICAL AND CULTURAL ASPECTS OF THE UTERUS

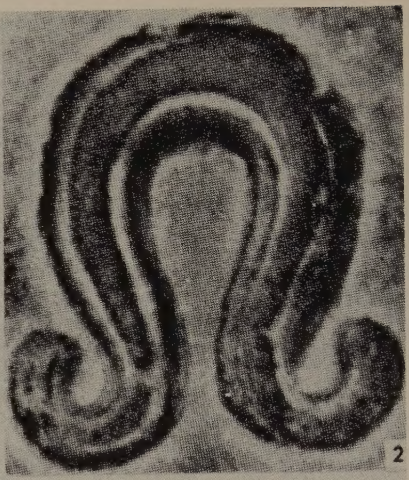
By Alfred Plaut
Armed Forces Institute of Pathology, Washington, D. C.

It has been known for many generations that the heart is a muscular pump, but it still remains the seat of emotions in our symbolism. In the same way, the uterus retains much of its age-old symbolic meaning, no matter how much function is transferred by knowledge to the ovary and other endocrine glands.

Even today there are people who do not know about the uterus as a separate organ. The Sinangolo in British New Guinea believe that conception takes place in the breasts and that the fetus later on falls down into the abdomen.[8] A Papuan drawing (FIGURE 1) of a pregnant woman shows a single large body cavity, four fifths of which is occupied by an outlined area with the infant in it. It reminds one of the way children draw the stomach when they picture a man eating. Old Chinese writings do not indicate knowledge of the uterus as a separate organ, and old Hindu books record the belief that conception and fetal development take place in a portion of the abdominal cavity.[9] The symbol of the Sumerian birth goddess Nintu (FIGURE 2), however, looks like a simplified representation of the uterus,[2] and the Egyptian hieroglyph SA or Ankh (FIGURE 3), which is often used ornamentally, recalls the shape of the human uterus. Its use on the figure of the birth goddess Taurt (FIGURE 4), in my opinion, strengthens this interpretation. Information transmitted to us about the Egyptian techniques of embalming leaves doubt as to the extent of the anatomical knowledge of the pelvic organs obtained during these procedures. Hippocrates and his pupils may never have seen a human uterus. Aristotle stated that he had no knowledge of the inner organs of man, only of animals.[13] This explains the pluralic forms of some Greek words for uterus, and also the statement that it has numerous circumscribed widenings at different distances from the vulva. And the uterine "cornua," which occur in most old descriptions, reflect the aspect of the animal organ. Pictures of the uterus with seven cavities (FIGURE 5) were published again and again despite the existence of more correct drawings. The Italian anatomist Berengario da Carpi (1470–1530) finally wrote, "*Est purum mendacium dicere quod matrix habeat septum cellulas*" (it is a sheer lie to say that the uterus has seven cavities). In his picture, a woman points a finger at her own unilocular uterus, which lies on a table, putting her foot contemptuously on books, the carriers of wrong traditions.

Anatomical knowledge did not progress steadily through the centuries of known history. The oldest picture of the uterus of which I know (FIGURE 6, Ninth Century) is better than many later ones, and it is so, paradoxically, because the description it follows is older by seven centuries—that given by Soranus of Ephesus about 100 A.D. Soranus, whose book on women's diseases reads "more like the 19th century than like the second,"[3] quite appropriately compared the shape of the uterus to a cupping vessel, and the names used for





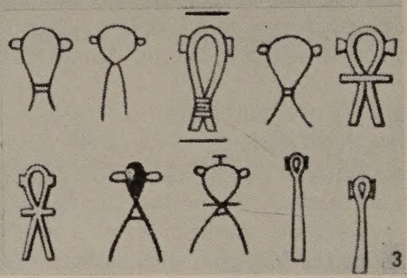


FIGURE 1. Papuan drawing of infant *in utero*. From P. Diepgen, 1937, in *Handbuch der Gynäkologie*. Courtesy of J. F. Bergmann, Verlag. Munich, Germany.

FIGURE 2. Uterine symbol from a Babylonian boundary stone. From A. A. Barb. 1953. *Diva Matrix. Jour. Warburg and Courtauld Institutes. 16:* 193–238. Courtesy of the Warburg Institutes. London, England.

FIGURE 3. Various shapes of the hieroglyph SA (Ankh). Compare the one marked with that shown in FIGURE 4. From A. A. Barb, 1953.

the parts of the uterus in this picture come much nearer to the ones used today than do those in books from the Middle Ages and the Renaissance. Most pictures do not reflect observation of the human organ, but either of animal organs, or, in true scholastic style, of bookish tradition without observation. Soranus, who wrote even about amputation of the prolapsed uterus, knew that the uterine cavity was single, but illustrations up to the Sixteenth Century give seven uterine chambers (FIGURE 5). Leonardo da Vinci indicated loculation in his early drawings (FIGURE 7). About half a century ago the physiologist Max Verworn,[17] spoke of physioplastic and ideoplastic art. In the first, the action of the creating hand comes straight from the visual apperception while, in the latter, ideas and concepts are interposed. Prehistoric cave drawings are the best examples of physioplastic art and, without turning to contemporary nonobjective art, one could hardly find a more ideoplastic picture than the Thirteenth Century drawing shown in FIGURE 8.

Sudhoff,[15] in discussing a Persian picture of a pregnant woman, speaks of

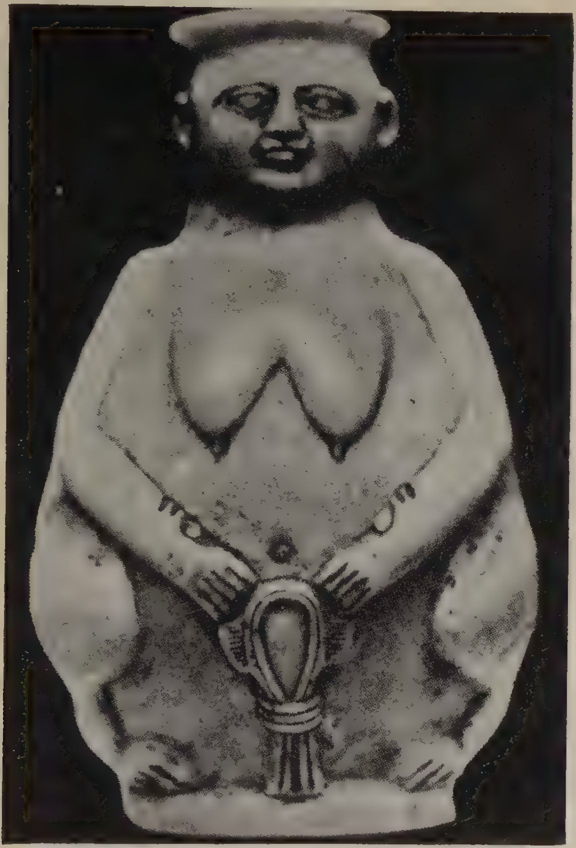
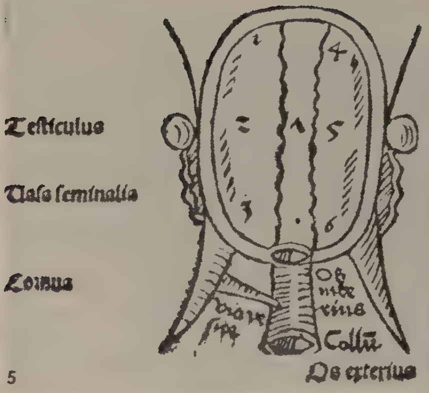


FIGURE 4. Egyptian vessel in the shape of the birth goddess Taurt. Compare with FIGURE 3. From A. A. Barb, 1953.

observation of nature on the one hand and tradition on the other. One wonders about the balance of the two methods in the student's classroom sketch of 1304 (FIGURE 9). The Soranus shape appears in schematic fashion in the beautifully illuminated Latin codex Monacensis Nr. 161, which dates from the Thirteenth or Fourteenth Century. In one of its pictures, which shows twins in podalic position, concentric lines might vaguely suggest membranes. But the graceful leaf ornaments (FIGURE 10) drawn on the same page cannot, by any stretch of the imagination, be equated with an anatomical structure. Corpus and cervix in their relation to the vagina are well shown in the Ketham picture of 1495 (FIGURE 11) and in some of Leonardo's drawings that date from about the same time (FIGURE 12), but they all have baffling lines that extend obliquely from body and cervix. The various names given these lines tell us little about the anatomical counterparts. In studying them we must remember that the nature of the Fallopian tubes was unknown. In Leonardo's "great situs" two pipes radiate obliquely upward and downward on



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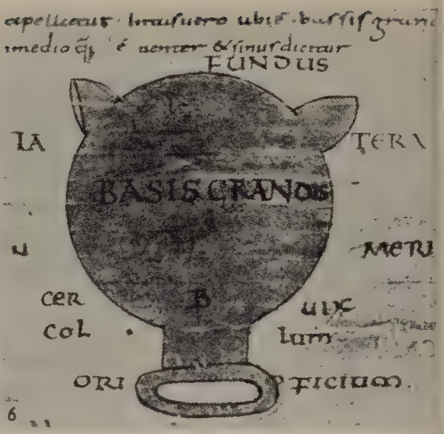

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FIGURE 5. Mundinus-type uterus from Magnus Hundt's *Antropologium*, 1501. The "seven cavities" are indicated by numerals. The ovaries are called testicles. Note the *cornua*. From F. Weindler. 1908. *Geschichte der Gynäkologisch-anatomischen Abbildung*. Verlag Zahn und Jaensch. Dresden, Germany.

FIGURE 6. Oldest known anatomical drawing of the uterus, Ninth Century, Soranus shape. From P. Diepgen. 1937. *Geschichte der Frauenheilkunde*. Veit-Stöckel's *Handbuch der Gynäkologie*. Vol. 12, pt. 1. J. F. Bergmann. Munich, Germany.

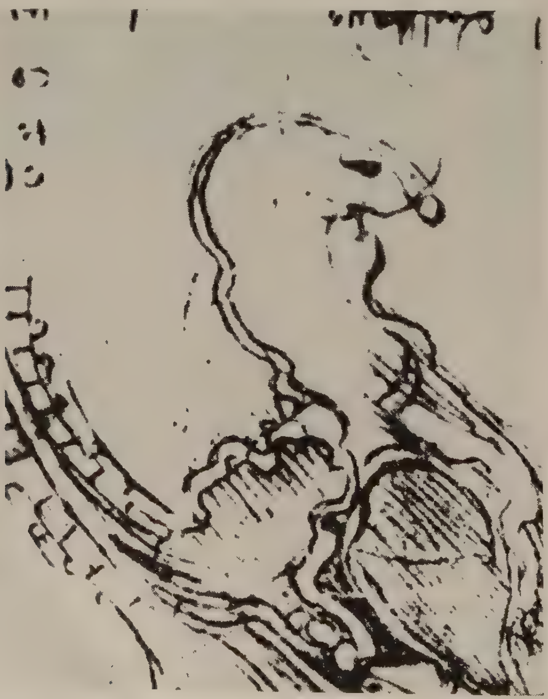

FIGURE 7. Partial view of Leonardo's coition picture. It shows the supposed duct that carries the menstrual blood as milk into the breast. This imagined structure is absent in Leonardo's later drawings. Note the loculation of the uterus. From G. Peillon. 1891. *Étude historique sur les organes génitaux de la femme, depuis les temps les plus reculés jusqu'à la renaissance*. O. Berthier, Paris, France.

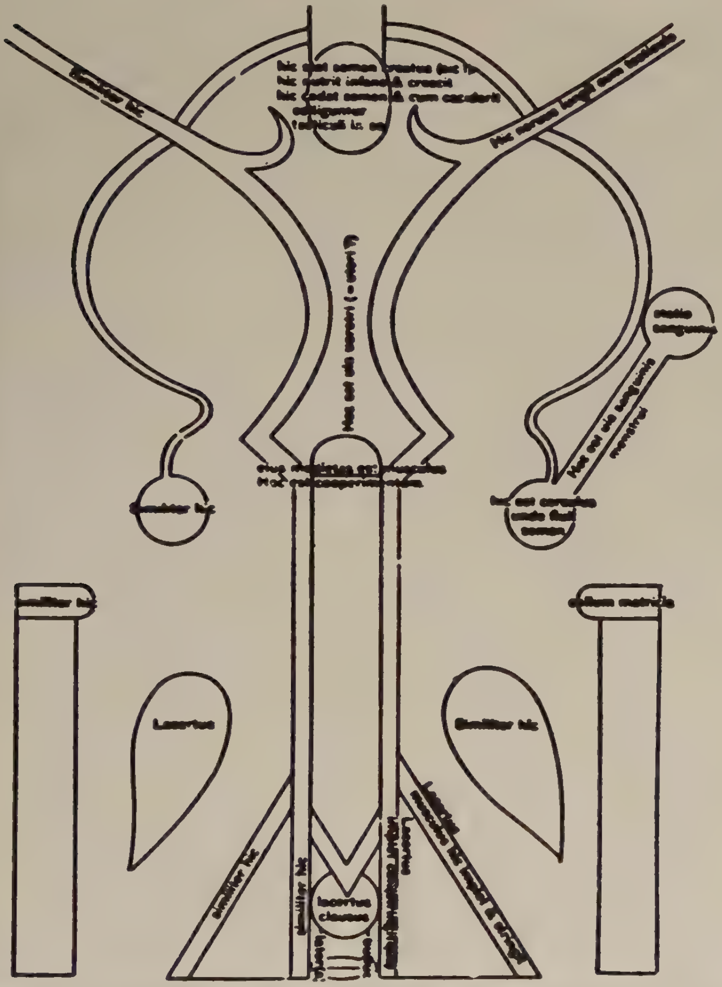

FIGURE 8. Thirteenth Century drawing of the uterus and vagina. A good example of representation according to preconceived ideas, not to observation. From Ch. Singer. 1915. Proc. Royal Soc. Med., London 9: 43–47.

either side. In the Ketham picture they curve upward; in the Mundinus-type uterus (FIGURE 5) from Magnus Hundt's *Antropologium* (about 1500) thin ones reach up at an acute angle, while broader ones labeled "cornua" extend down from the lower portion. In Hans Guldenmundt's picture from 1539 (FIGURE 13), *cornea*, obviously meaning cornua, is written on broad, scimitar-shaped formations that extend upward from the ovaries along the edges of the uterus. The belief that the ovaries (called testicles*) do not contribute to the formation of the embryo prevented a study of their connec-

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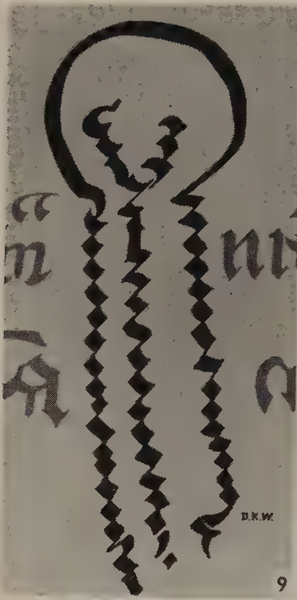
* The use of the same word for male and female structures still exists, as in the English terms seed and sperm, and in the German word *Samen*.



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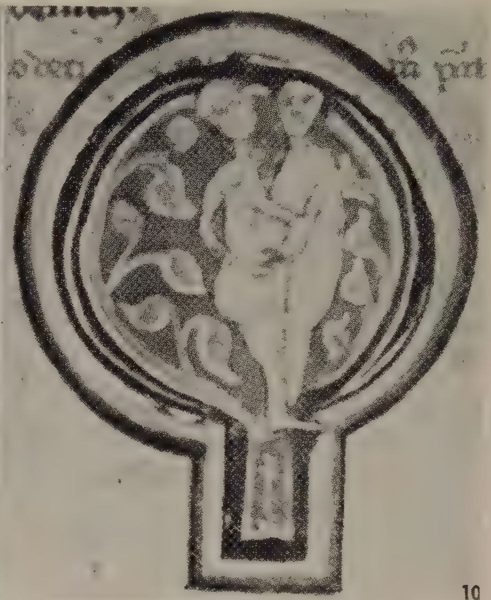

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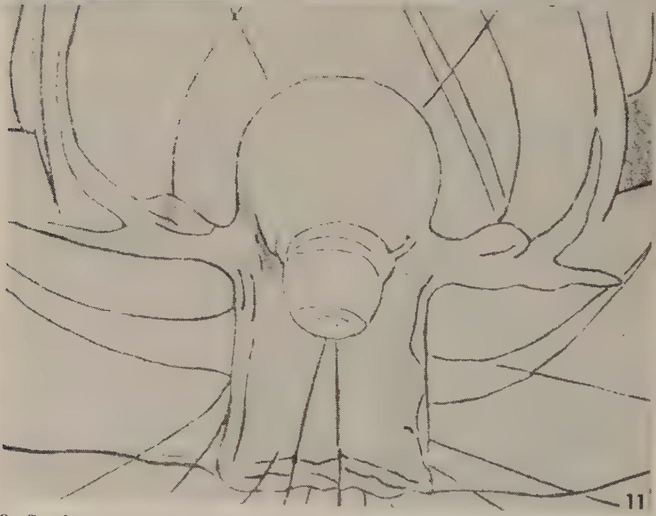

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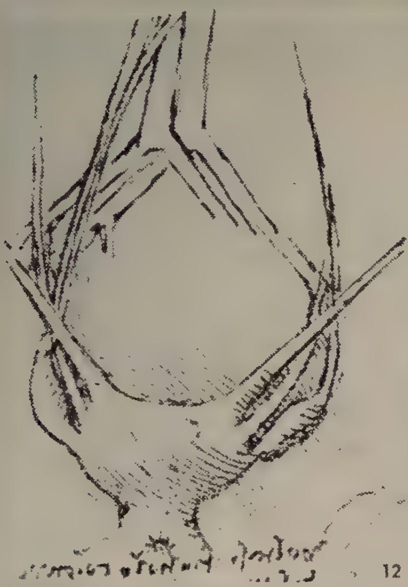
FIGURE 9. Student's classroom sketch of the uterus, 1304. Note the overlong lower portion which obviously represents the cervix and vagina. From Weindler. 1908. *Geschichte der Gynäkologisch-anatomischen Abbildung*. Verlag Zahn und Jaensch. Dresden, Germany.

FIGURE 10. Uterus with twins. The concentric lines may or may not represent membranes. The leafy pattern may be purely ornamental. Codex Monacensis, 13th–14th century. From Weindler, 1908.

FIGURE 11. Uterus with opened vagina. Ketham, 1495. An unusually good picture of the uterus itself. But note the disturbing lines of the appendages. From Weindler, 1908.



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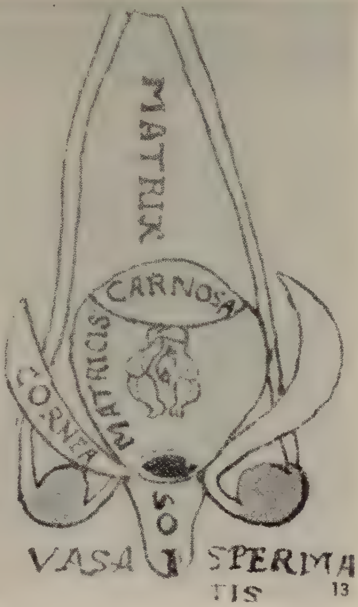

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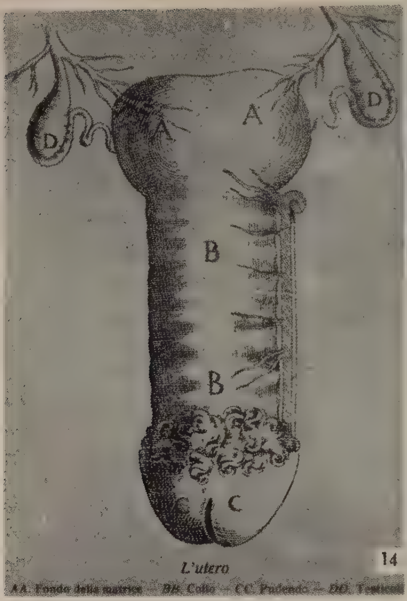
FIGURE 12. Sketch of the uterus by Leonardo da Vinci. The shape of the uterus is well given, but the appendages are not drawn to nature. From E. Belt. 1955. *Leonardo the Anatomist.* Courtesy of the University of Kansas Press.

FIGURE 13. Uterus figure by Hans Guldenmundt, 1539. The structures labeled "cornea" resemble the wings on Gnostic gems. Compare with FIGURE 31. From A. A. Barb. 1953.

tion with the uterus (Rufus had known about the tubes opening into the uterus, but this was forgotten). The ovaries were often pictured as being low down and near the midline (FIGURES 5 and 13). Some thought they were secondary sex glands that furnished a fluid to call forth the female orgasm, others took them to be superfluous, like the nipples of the male. But the appendages, tubes, ligaments, and vessels stimulated the imagination of anatomists.

The erroneous beliefs that distorted the anatomical pictures were concerned mostly with physiology. It was assumed that the semen of the male was deposited in the uterus during coition, and it followed naturally that the lower part of the uterus was pictured as wide enough for immission of the penis (FIGURE 9). Accordingly, the vagina and uterus were shown with one canal of even width. Rufus of Ephesus[18] (around 100 A.D.) had correctly regarded the uterus and vagina as separate organs, but this, too, was forgotten. Some circumvented the difficulty by assuming that the cervix opened up during intercourse. Little wonder then that one cannot accept the anatomical designations at face value, but must try in the single instance to discover what was meant by collum, cervix, matrix, and other terms. In a letter


D
A
A
D
B
B
C
C
L'utero
14
AA. Fondo della matrice — BB. Collo — CC. Pudendo — DD. Testicoli

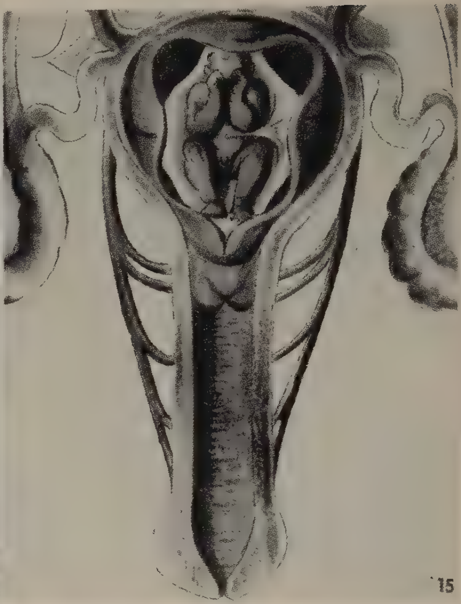


FIGURE 14. Phalluslike representation of female genitals. Scipione Mercuri, 1596.

FIGURE 15. Female organs with pregnancy, by Georg Bartisch, 1575. "Assimilation" to the male organ. The excessive length of the vagina reflects the age-old uncertainty about cervix and vagina. From Weindler. 1908. *Geschichte der Gynäkologisch-anatomischen Abbildung.* Verlag Zahn und Jaensch. Dresden, Germany.

addressed to Rubens, the word vulva is used, while the sketch in his answering letter shows a uterus.

This merging of vagina and uterus fitted another preconceived idea, namely, the homology of the male and female organs. The ovaries are called testicles in many illustrations. It is hard to believe that the man who drew the picture labeled *L'utero* for Scipione Mercuri's book (1596) did not have the phallus in mind (FIGURE 14). The same applies to the picture of a uterus in an English edition of Vesalius. A famous French surgeon, Guy de Chauliac (about 1300 1368),[14] gives a careful description of the homology of male and female parts. While it is difficult to be sure about the meaning of his old French terms, an abbreviated translation might read as follows: "The uterus is like a penis turned inside out . . . it has in its lower part two arms with the testicles . . . like the scrotum, . . . a common body in the middle . . . a collum with a canal in it like the shaft . . . and the vulva is like glans and preputium." Two colored pictures from the *Kunstbuch of Georg Bartisch*, 1575, illustrate the same idea (FIGURE 15). Leonardo, giving a diagrammatic sketch, describes the homologies in detail, correlating the uterus with the seminal vesicles.

The ideas about fetal nourishment also were reflected in anatomical representations. It seemed reasonable to assume that the infant must have practiced sucking *in utero*, since he knew how to do it when born. Thus there must be something akin to nipples in the womb, and the *Anatomia Curiosa* of

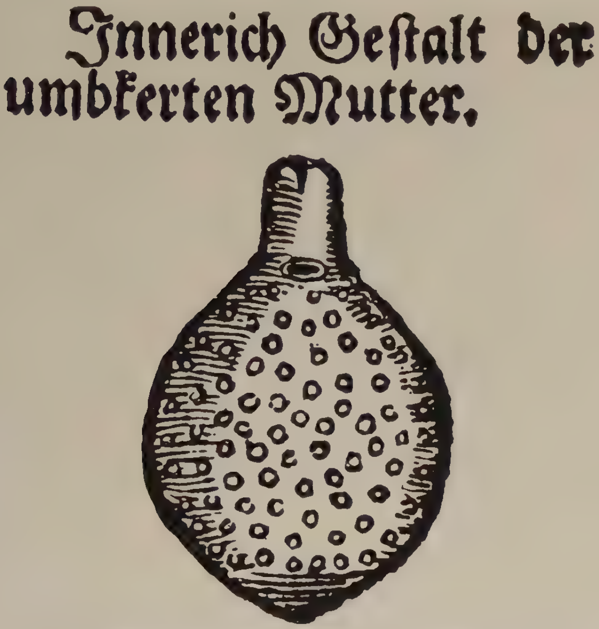


FIGURE 16. Inverted uterus, by Antonius Novarini, *Anatomia curiosa,* 1682. The many small circles probably represent the so-called cotyledons.

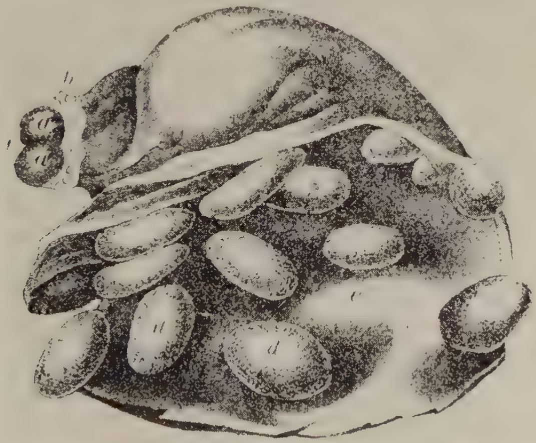


FIGURE 17. Pregnant uterus of a cow, with cotyledons. From *Special Report on Diseases of Cattle*, Bureau of Animal Industry, 1942. U. S. Government Printing Office.

Antonius Novarini, 1682 (FIGURE 16), shows numerous small round structures on the endometrial surface. Such structures, called cotyledons, do exist in the uterus of the cow (FIGURE 17). Since Hippocratic times, the retained menstrual blood was believed to be the food of the fetus. After delivery it was

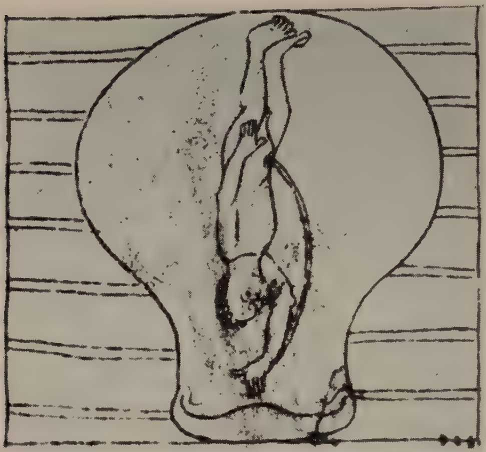
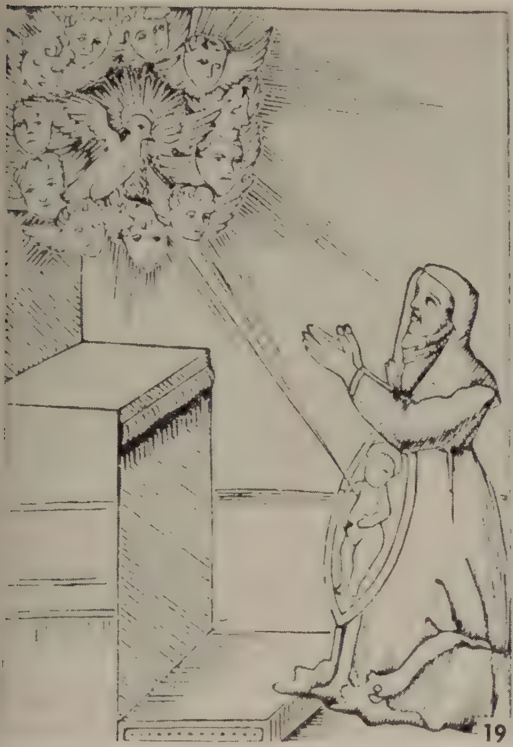


FIGURE 18. Naturalistic sketch of a pregnant uterus showing the umbilical cord (Fourteenth Century). From R. Salomon, 1936. *Opicinus de Canistris. Studies of the Warburg Institute, I B.* Courtesy of the Warburg Institutes, London, England.

conveyed to the breasts, and there, in the form of milk, continued to nourish the infant. Therefore, a canal must exist between the uterus and the breast. One of Leonardo's earlier drawings shows this canal (FIGURE 7). Probably superior epigastric and internal mammary vessels formed the anatomical basis of this assumption. Old German writings reveal the term *Milchader* (milk vessel); perhaps the word *Milchbrustgang*, the German term for the thoracic duct, harks back somehow to forgotten beliefs, and is not based entirely on the milky aspect of lymph.

The Middle Ages were preoccupied less with anatomy than with religious mysticism. The drawings of Opicinus de Canistris, an Italian cleric who lived in Avignon, France, at the time of the papal schism, are difficult to interpret because Opicinus was schizophrenic. But one of them (FIGURE 18) is unusually accurate as to the shape of the uterus and of the term fetus; it even shows the seldom depicted umbilical cord. In another drawing, additional contours inside the uterus defy interpretation and, in a third, the infant stands in a circle. Opicinus' source for the anatomically correct drawing is unknown to me. A simple mostly oval outline was used in religious pictures to indicate the uterus near term, as in a German picture (1524) entitled "The Holy Virgin as Vessel of the Divine Child" (FIGURE 19).[11] A highly artistic sublimation of the theme indicates the pregnant state of Elizabeth and Mary in a graceful Fourteenth Century visitation group from near the Lake of Constance (FIGURE 20).

Votives and amulets furnish nonanatomical representations of the uterus. The hopeful or thankful prayer of suffering people was reinforced by placing a picture of the diseased organ near the altar. Many clay votives have been found in Italy; one from the Greek island of Kos has an added pear-shaped structure which may represent the urinary bladder (FIGURE 21). Some have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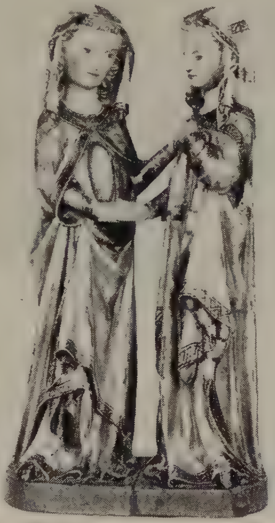
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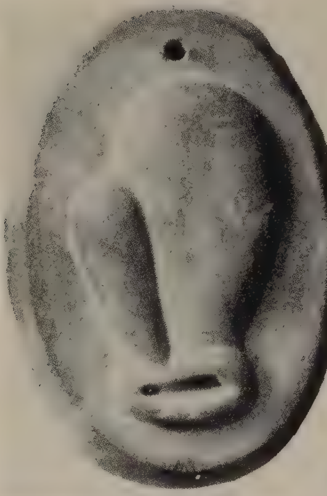
FIGURE 19. The Virgin Mary as vessel of the Divine Infant, 1524. The uterus is indicated by a shieldlike double contour. From C. G. Jung, 1944. *Psychologie und Alchemie.* Courtesy of Rascher Verlag. Zürich, Switzerland.

FIGURE 20. Visitation group, ascribed to Master Heinrich of Constance, about 1310. The oval shields in the lower sternal area may indicate the spiritual nature of the pregnancy. Courtesy of the Metropolitan Museum of Art, New York, N. Y.

transverse ridges and grooves the meaning of which has been much discussed, especially in relation to the rugae of the vagina (FIGURE 22). Further, there is no agreement about lengthwise markings in the lower portion and single or multiple lower openings. The above-mentioned failure to distinguish between uterus and vagina makes interpretation difficult. I agree with Sudhoff[15] that detailed anatomical interpretation should not be attempted. Sometimes umbilicus, uterus, and vulva are shown in semidiagrammatic fashion (FIGURE 23). The markings on the votives, whose meaning must have been obscure to the artisans, later were ornamentalized (FIGURE 24).

To my knowledge, the uterus is the only organ represented in the votive as an animal, namely the toad. Toad-shaped votives[5] existed in prehistoric times and still exist (FIGURES 25 and 26). Thirty years ago they were used in Bavaria, Germany. A German church record of votives covering 500 years lists toad votives only for uterine complaints. This brings us to the question



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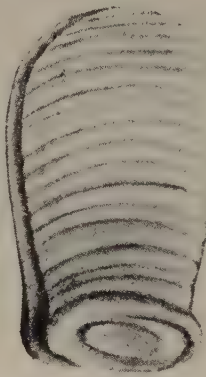

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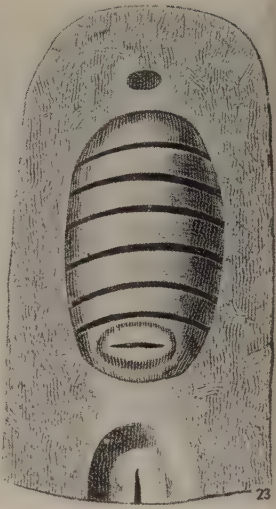

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FIGURE 21. Uterine votive from the island of Kos. The smaller pear-shaped structure may represent the urinary bladder. From Diepgen, 1937, in *Handbuch der Gynäkologie*. J. F. Bergmann. Munich, Germany.

FIGURE 22. Uterine votive with transverse ridges. From the Island of San Bartolomeo, in the Gulf of Trieste in the Adriatic Sea.

FIGURE 23. Old Italian votive, showing uterus, vulva, and umbilicus. From Weindler. 1908. *Geschichte der Gynäkologisch-anatomischen Abbildung*. Verlag Zahn und Jaensch. Dresden, Germany.

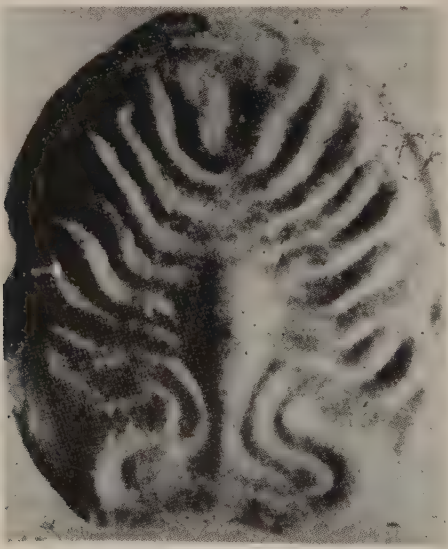
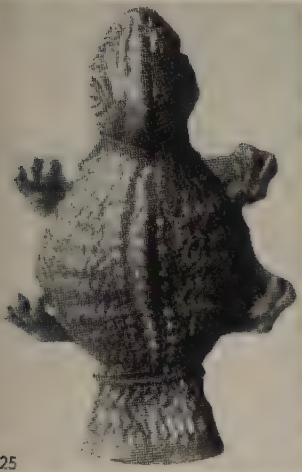


FIGURE 24. Ornamentalized uterine votive.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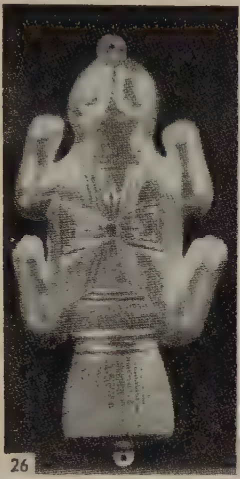
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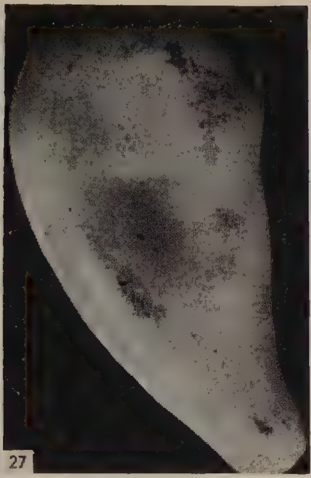
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FIGURE 25. Toad-shaped uterine votive. From E. Holländer, 1912. *Plastik und Medizin.* Courtesy of Verlag Ferdinand Enke, Stuttgart, Germany.

FIGURE 26. Silver toad votive with cross on back. Bavarian. From Kriss, R. 1929. *Das Gebärmuttervotiv. Ein Beitrag zur Volkskunde nebst einer Einleitung über Arten und Bedeutung der Deutschen Opfergebräuche der Gegenwart.* Courtesy of Benno Filser Verlag. Augsburg, Germany.

FIGURE 27. Realistic waxen uterine votive. Austrian, ca. 1900. From R. Kriss, 1929.

of why only the toad evolved as a symbol. Some explain that the toad always was considered powerful in magic, a *Seelentier* (soul animal); others see a similarity between the shape of the uterus and that of a plump toad. According to a Twelfth Century story, the Emperor Nero wanted to know how it felt to be a pregnant woman, so his physician gave him a powder that made a toad grow in his belly, and he looked like a pregnant woman. Whether or not the toad was meant originally to represent the uterus, the women who offered the votives believed it, and when, with the spread of medical knowledge, this belief was shaken, occasionally a more realistic votive (FIGURE 27) was substituted. In the 1920s the toad votive and the realistic one were seen side by side in Austrian churches.[13]

We use the word hysteria without considering its origin from Greek *hystera*, the uterus, or merely with the psychosomatic power of the female reproductive apparatus in mind. In Greek medical writings, however, there are statements such as "the uterus causes a thousand evils," and even "the uterus is the cause of all diseases."[8] When one tries to understand how such ideas could arise, it comes to mind that to the unsophisticated the womb, which is the source of life, must be more alive than other organs, a living being itself, and hence endowed with the power of moving around like an animal. No other organ, to my knowledge, has been credited with that power. The Latvian and Silesian belief that the uterus can leave the body and walk cross-country[4, 16] seems to be rare, but the belief that the uterus may roam freely within the body has been shared by many people in many epochs. The Egyptian papyrus Kahum (Third Millennium B.C.) discusses and prescribes treatment for diseases of the throat and of the eye that are caused by ascending of the uterus. Following the custom of historians, we may assume that the Greeks took the idea over from Egypt but, since aborigines in Malayan islands have almost identical beliefs,[1] we cannot know in how many parts of the world the idea of the wandering uterus may have arisen independently. Plato, in the dialogue *Timaeus*, calls the uterus an animal. His source could have been Socrates, whose mother was a midwife, or he could have heard of it while traveling in Egypt. Galen and Soranus of Ephesus did not believe it and Soranus protested against it, but Aretaeus of Cappadocia (about 300 A.D.), one of the best informed physicians of antiquity, writes as follows: "In the middle of the flanks of women lies the womb, a female viscus, closely resembling an animal, for it is moved of itself hither and thither in the flanks, also upwards in a direct line to below the cartilage of the thorax, and also obliquely either to the right or the left, either to the liver or the spleen; and it likewise is subjected to prolapses downwards, and in a word is altogether erratic. It delights also in fragrant smells and advances toward them, and it has an aversion to fetid smells and flees from them; and on the whole the womb is like an animal within an animal. Hence it may be suddenly carried upwards, compressing the lungs, heart, diaphragm, liver, intestines, obstructing the breathing and speech, and compressing the carotids and causing stupor."[7] According to the Hippocratics, choking is one of the most important symptoms of its malposition; this makes us think of the globus hystericus. Widows and spinsters are most endangered, since this animal, the uterus, becomes wild when not fed with male sperm. Intercourse and pregnancy were prescribed, and also the smelling of fetid substances to drive the uterus down and vaginal application of aromatic wine to lure it back. The same concept of the uterus is to be found also in Arabic writings of the Ninth Century, and European medical books perpetuated it to the end of the Seventeenth Century. Consciously or not, the great role malpositions of the uterus played in Nineteenth Century gynecology may have harked back to such ancient beliefs. This is farfetched, but the stubbornness of such a belief is shown in folk medicine.

In East Prussia and southward, down to Upper Silesia, *Himmelsbriefe*[4] (letters from heaven) are sold by itinerant peddlers to this day. In previous centuries they were widespread in Germany. The letters were said to have dropped from heaven at Bethlehem or some other holy place. This fraud is more than one thousand years old. St. Boniface, the Irishman who converted the German tribes, complained about it in a letter to the Pope. The *Himmelsbriefe* contain a great variety of magic spells against colicky pain, addressed to the *Mutter* which is an abbreviation for *Gebärmutter* (uterus). The fact that *Mutter* means mother gives the wording of the charms overtones that cannot be rendered in English. They are printed in German or in one of the Slav tongues of the region. They are not meant as black magic; the *Mutter* is conjured in the name of God, the Trinity, or the Virgin. Various names are used for the uterus; one charm contains sixteen of them. These names are combined words ending with *Mutter*; they are religious or anatomical, or are named according to function or to designate symptoms. The uterus is conjured to go back to the place where Jesus Christ has ordered her to be, or the charm reads: "Go and sleep in thy chamber where God has created thee." Often repetition of the charm is prescribed, alternating with the Our Father. The *Mutter* is told that masses have been said, that the Gospel has been read, that church bells have been ringing, and that therefore she must return to her place and stop torturing the woman. Long Latin incantations were written in the famous monastery of St. Gallen in Switzerland in the Eighth Century. One from the Fourth Century asks the uterus: "*Quid irasceris, quid sicut canis iactas te, quid sicut lepus resilis? Quiesce intestinum*" (Why art thou mad, why dost thou throw thyself around like a dog, why dost thou jump like a hare? Keep my innards quiet)." The word *intestinum* in this context means inner abdominal organs in general, not intestine as we use the word today. It was forgotten sometimes that the charms were meant for women only, and a medical dictionary of 1725 makes fun of ignorant male persons who complain that they have *eine böse Mutter* (a bad uterus). The obvious discrepancy made people coin the term *Bärvatter*; *Vater* means father.

The anatomical drawings and the votives are not the only known old representations of the uterus. In the early centuries of our era there was a strong widespread religious movement called Gnosticism, a mystical religion with its roots in oriental mythologies and burdened with an endless variety of spirits and demons. The Church fought with energy against the heresy of Gnosticism. The uterus, called metra or matrix, plays a role in Gnostic writings; there is the idea of a divine primeval womb, which probably came from Orphic sources out of Asia Minor. Gnosticism assembled and assimilated many deities, among them the Egyptian Isis. In the Second Century A.D., Apuleius (author of *The Golden Ass*) described an emblem carried in the procession of Isis, applying terms that were used to designate the parts of the uterus. Metra, according to a Gnostic cosmogony, was born from the union of Darkness and Spirit. The womblike shape of heaven and earth resulted from the first intercourse. More than a thousand years later, Paracelsus wrote that God had formed heaven and earth into a womb (matrix) in which Adam was conceived; just as man lives in this womb of the outer world, so the unborn child lives under the firmament of the mother's womb. The water over which the spirit of God moved before creation was the womb in which heaven and earth were created.[2] The kinship of this with Gnostic writings is unmistakable, although we do not know what Gnostic books Paracelsus may have read. Again, more than a century later, Sir Thomas Browne, in *Religio Medici*, called the uterus "that other world, the truest microcosmos," at the same time proffering the modern thought that the fetus *in utero* is subject to exogenous diseases.

Gnostic beliefs about the uterus found expression in amulets that were collectors' items in the Seventeenth Century and sometimes were faked. Most of them are intaglios. It is a difficult but enticing task to unravel their meaning and to relate the shapes carved on them to anatomical structures. Essentially, I follow the interpretations by Barb[2] and by Bonner.[6] The shape of

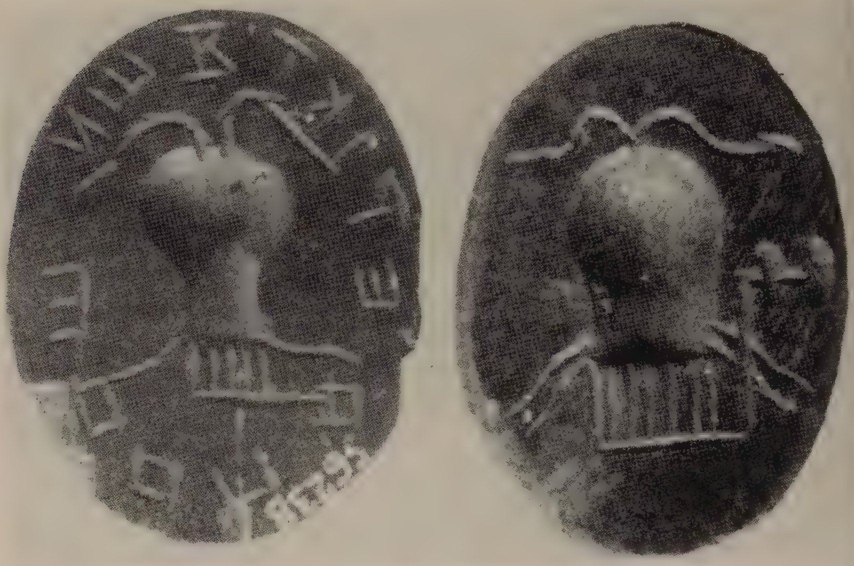
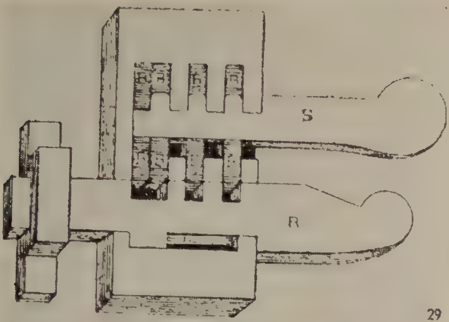


FIGURE 28. Two Gnostic uterine gems with filaments and with the key. In one, the handle of the key is shown. From A. A. Barb. 1953.

the human uterus with a more or less distinct cervix is easily recognized in FIGURE 28, and this interpretation, in my opinion, has definitely replaced earlier ones. The womb sometimes occupies the entire field of the gem; sometimes it is under the feet of a god or held in the god's hand. These deities are Egyptian in style, but the name of the Jahve of the Old Testament also appears together with mystic symbols. Parallel lines under the cervix on some gems could not be explained were it not for others on which they appear as the bit of a key (FIGURE 28). The identity of this detail and the bit and handle of antique keys may be seen by comparison with FIGURES 29 and 30. I do not know whether the key was meant to unlock, meaning to combat sterility, or to lock, meaning to prevent conception, but there is good evidence that when the gem was worn by a man it was supposed to lock his wife or mistress against the sexual approach of any other man.

On the gem in Rubens' possession the uterus has wings, which the painter compared to butterfly wings (FIGURE 31). Divine symbols, the phallus, for example, were often shown winged. The structures labeled "cornua" in some medieval and Renaissance anatomy books closely resemble the wings on this gem and, in the virtual absence of unprejudiced observation, may have been suggested by them (FIGURE 13). Thin, filamentous, straight, or wavy lines run outward from the upper and lower portions of the uterus (FIGURE 28). They remind one of the incomprehensibly willful ways in which appendages and uterine vessels were pictured in anatomical drawings up to the time of



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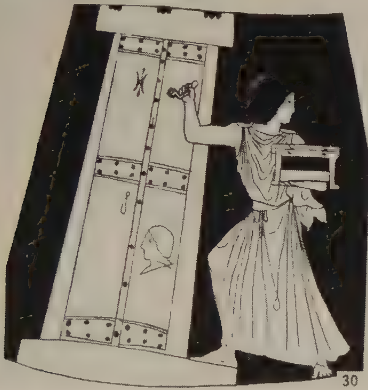

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FIGURE 29. Model of a Greek key. Compare with the vertical lines on the Gnostic gems. From H. Diels, 1914. *Antike Technik.* Courtesy of Verlages, B. G. Teubner, Stuttgart, Germany.

FIGURE 30. Girl opening a door with a key. Greek vase in Berlin Museum. From H. Diels, 1914, *Antike Technik.* Courtesy of Verlages, B. G. Teubner, Stuttgart, Germany

Leonardo and in his earlier work (FIGURE 13). Little wonder that they sometimes were depicted as snakes, with their magical and sexual connotations. There may be few or many of the lines; in the latter case the uterus is said to be of the octopus type. In one most peculiar Gnostic gem (FIGURE 32) the uterine symbol has become a funnel with transverse ridges, as seen on the old Greek and Italian votives, together with a liberal array of long filaments. The funnel surrounds a woman from the waist to the knees.

The uterus (matrix), like other divinities, was sometimes shown upon an altar (FIGURE 33). In Egypt the face of the mother goddess Hathor was put within the uterine symbol (FIGURE 34), which resulted in its being called Hathor's hairdress. In an Etruscan terra cotta (FIGURE 35), the embellished matrix has become the frame for a head. The "diva matrix" survives to this day as an ornamental frame of forgotten origin. The octopus type of symbol appears in medieval Byzantium as a human face from which serpents radiate. They are inscribed with a message to the uterus: "That restless beast whose

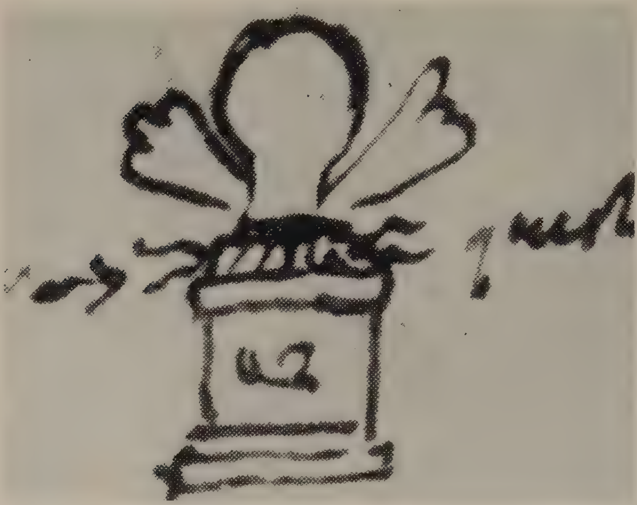


Figure 31. Rubens' sketch of a winged uterus on altar. Compare with figure 13. From A. A. Barb, 1953.

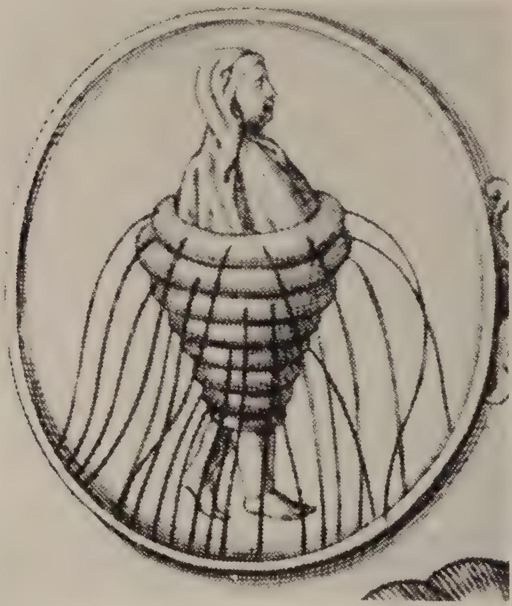
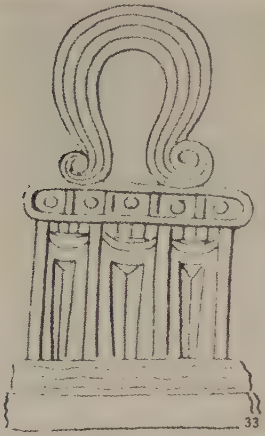
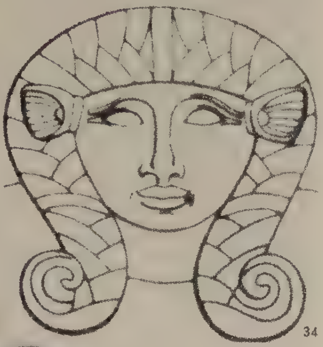


Figure 32. Gnostic gem from Macarius Chiflet. See text. From A. A. Barb, 1953.





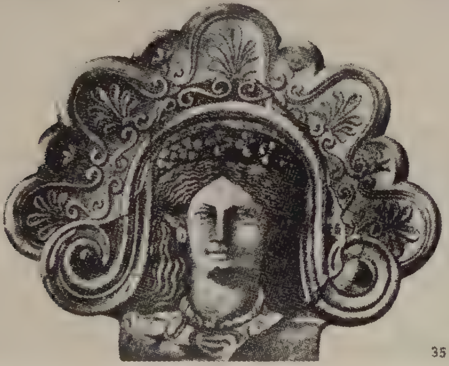


Figure 33. Divine uterine symbol (Ninharsag from Babylon) on altar. From A. A. Barb, 1953.

Figure 34. The face of the mother goddess Hathor surrounded by a uterine shape. From A. A. Barb, 1953.

Figure 35. Etruscan female head surrounded by an ornamentalized uterine symbol, which recurs in the outer frame. From A. A. Barb, 1953.

rage must be quieted by magic." Thus the Medusa, or Gorgon, that horrifying woman's head with snakes for hair, is also related to the uterine symbol. The psychoanalist Ferenczi, by analyzing dreams and associations, has come to interpret the Medusa as a sexual symbol. As Bonner[6] nicely remarks, the Gnostic representations of the uterus are truer to nature than our customary symbol of the heart.

The alchemists of the Middle Ages and of later centuries vie with the Gnostics in mystical ideas about the uterus. The womb is the vessel in which the child is created; and the vessel in which the alchemist makes a human being, a homunculus, resembles the uterus (FIGURE 36), as shown in the well-known picture from Alexander Röslin's *Rosengarten* (FIGURE 37). C. G. Jung[11, 12] includes this in the symbolism of the vessel.

It remains to mention the most puzzling of uterine symbols, the spiked ball of the Eastern Alps, especially of South Tirol, and also of Styria and Austria (FIGURES 38 and 39). No one, when looking at these objects, will think of the uterus, but thirty years ago the farmers' wives who put these votives next to the altar thought they represented the uterus and, according to the man who carved and sold them, they were offered for women's ailments. An old painting in the monastery of Andechs, south of Munich, depicts a woman kneeling before the Virgin Mary; the legend states that the woman was from the Tirol, had been suffering from the uterus (*muttersiech*) for a long time, and was cured after dedicating this picture. The spiked ball is shown. It is noteworthy that the patient was from the Tirol. In a corresponding Bavarian picture of 1811,[13] the toad is shown (FIGURE 40). The spiked ball was used for the "rising mother" (mother stands for uterus). "She rises with the spikes up to the neck but when one dedicates something one gets better." Here we have the globus hystericus. A satisfactory explanation for the transformation of the uterine shape into a spiked ball seems hopeless until we remember how the structures that radiate from the uterus have been overemphasized in anatomical drawings and on the Gnostic gems (FIGURE 28). The placing of a face in the center of the octopus-type uterine symbol probably led to the Medusa type with lines (snakes) radiating in all directions. This type was found mostly in the Balkans, and the fact that the spiked ball is found mainly in the southeastern Alps suggests a connection.

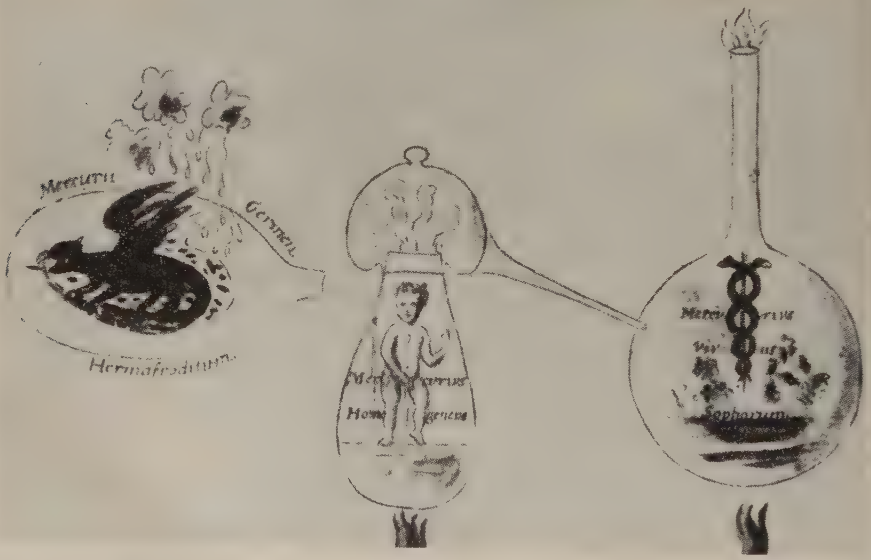


FIGURE 36. Alchemic apparatus with a uterus-shaped vessel. Turn book upside down and compare the central picture with the uterus in FIGURE 37. From C. G. Jung, 1944.

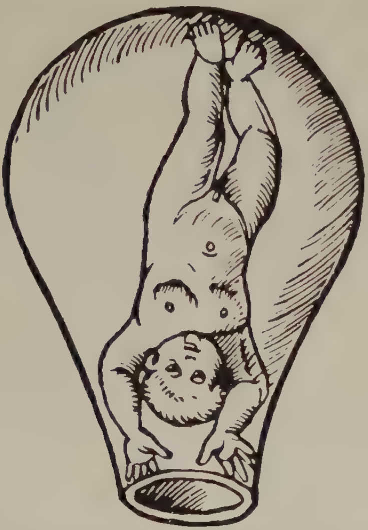
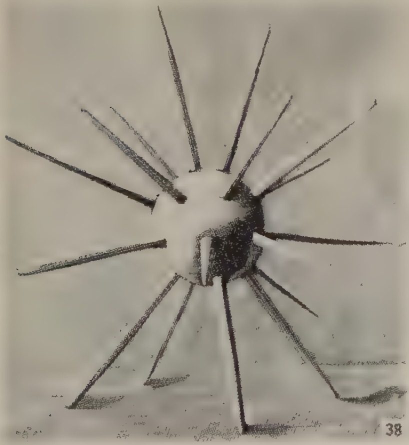


FIGURE 37. Uterus with full-term infant. From A. Röslin's *Rosengarten*, 1529. Compare with FIGURE 36.



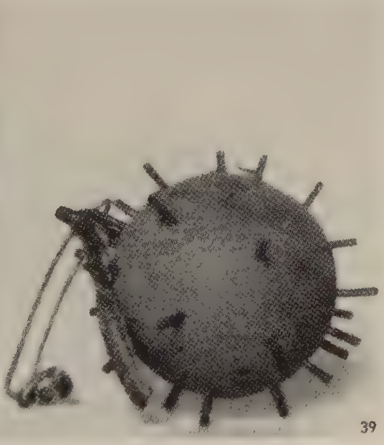


FIGURE 38. Wooden *Bärmutter* from Tirol, a uterine votive in the form of a spiked ball. From W. Hein,[10] 1900. *Die Opferbärmutter als Stachelkugel. Ztschr. d. Vereins für Volkskunde,* Zehnter Jahrg. **10:** 420–426.

FIGURE 39. Uterine votive, spiked ball with short spikes. From the Trentino. From Kriss, R. 1929. *Das Gebärmuttervotiv. Ein Beitrag zur Volkskunde nebst einer Einleitung Über Arten und Bedeutung der Deutschen Opfergebräuche der Gegenwart.* Courtesy of Benno Filser Verlag. Augsburg, Germany.

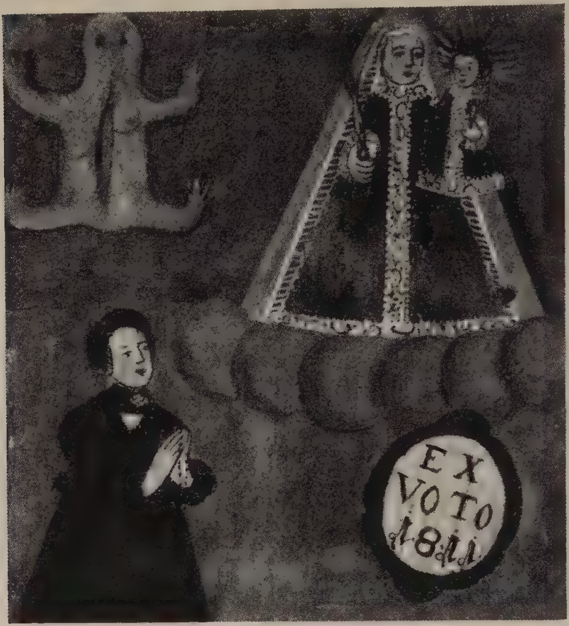


FIGURE 40. Votive picture with toad. Austria, 1811. From Kriss, R. 1929. *Das Gebärmuttervotiv. Ein Beitrag zur Volkskunde nebst einer Einleitung Über Arten und Bedeutung der Deutschen Opfergebräuche der Gegenwart.* Courtesy of Benno Filser Verlag. Augsburg, Germany.

One name given this form of the uterine votive is *Igelkalb* (*Igel* is hedgehog, *Kalb* means calf). The pregnant uterus of the cow, when inverted, was called *Igelkalb* because of the large cotyledons on the endometrium (FIGURE 17); with the above-mentioned elasticity of nomenclature, uterine appendages sometimes were called cotyledons also. As a last straw, it has been suggested that a poor linguist may have confused the words *hystera* (uterus) and *hystrix* (porcupine).

What has been discussed here represents only a fraction of today's knowledge concerning the cultural aspects of the uterus. Continued efforts in anthropology, archaeology, depth psychology, and comparative linguistics must bring out better the unique position the uterus occupies in the history of culture.

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EMBRYOLOGY OF THE UTERUS: NORMAL AND EXPERIMENTAL*

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This short embryologic introduction includes descriptive and comparative aspects of the subject only to the extent that they serve to point up the special character of the female gonaduct system or may establish time relationships between its step-by-step development and that of the sex glands, as well as of other organs. Main emphasis, however, is placed on the experimental analysis of mechanism of induction and differentiation. A reappraisal of the often discussed questions of permanence or reversibility of embryonic determination, of tissue and organ specificity, and of the causes of abnormal development may lead to some new concepts and generalizations.

Descriptive Analysis

Development of the primitive oviducts.† The question of embryonic origin involves the consideration of homologies on an evolutionary basis, as well as the direct analysis of the developmental process. To avoid a lengthy review of often confusing literature on the comparative anatomy and embryology, I refer here only to the fact that in selachians and amphibians the oviducts clearly arise in connection with the pronephros. It is not unlikely that in primitive ancestors sperms and eggs were conducted to the outside by segmental kidney tubules not especially adapted to this task (FIGURE 1*a*). Oviducts and seminal ducts of the higher vertebrates evolved from this condition by specialization, not as neoformations. Even the selachians show a higher differentiation; the mesonephros has assumed the function of sperm transport, while some pronephric elements specialize in the removal of the large eggs from the body cavity. This leads to a complete separation of the two systems. Early in embryonic development the pronephros loses its urinary functions and usually three of its nephrostomes fuse, forming a single larger funnel, the ostium of the oviduct. While the mesonephros takes over the excretory functions, the primitive nephric duct, through a process best characterized as lateral budding, separates into a mesonephric duct and an oviduct; the former serves the urinary function and in the male is also a seminal duct (FIGURE 1*b*).

Specialization advances further in amphibians. The urinary pronephric tubules differentiate very early, but the development of the funnels and tubes of the oviducts is considerably delayed. The oviduct field is the lower part of

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† In accordance with the recently accepted revision of the *Nomina Anatomica* (1956), the eponyms "Wolffian duct" and "Müllerian duct" will no longer be used. In their stead more descriptive designations are adopted, such as nephric, mesonephric, and deferent seminal duct, on the one hand, and oviduct, on the other. The term oviduct is used for the entire female gonaduct, from its funnel to the lower end where it meets the cloaca or the urogenital sinus.

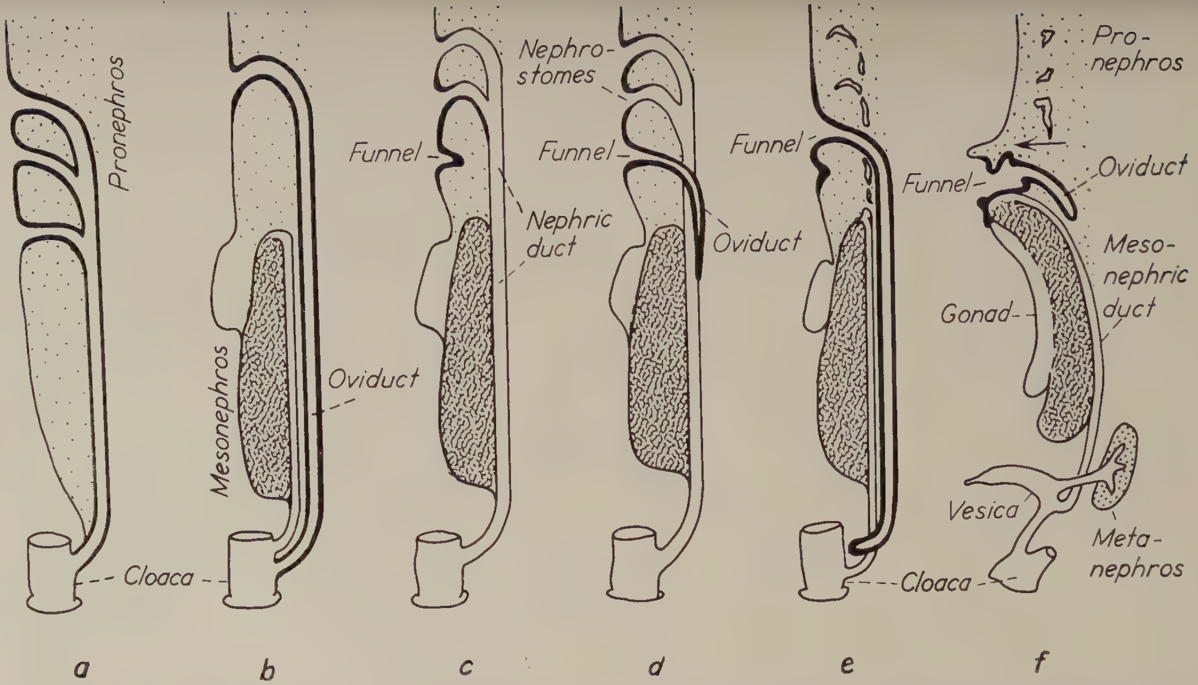


FIGURE 1. (*a*) Primitive condition of pronephros (embryonic stages of selachians and amphibians); (*b*) separation of primitive nephric duct into oviduct and mesonephric duct (selachians); (*c*) early stage of oviduct formation by invagination of oviduct field (amphibian larval stage); (*d*) downgrowth of oviduct along the mesonephric duct (in salamanders the tip is imbedded in the wall of the mesonephric duct); (*e*) postembryonic and prepuberal condition typical for most vertebrates: oviduct has reached the wall of the cloaca, the pronephros degenerating; (*f*) early oviduct formation in man (embryos of 14 mm., stage 29 to 30). Funnel of oviduct is under the pleuroperitoneal fold, shown by the arrow and, its fimbria is spread over the top of gonad and mesonephros.

the nephrostomic area, covering a large part of the pronephric eminence. It is a thickened and in later stages, a ciliated, epithelial plate and should be considered as a derivative of the intermediate or nephric mesoderm rather than of the lateral plate. By forming first one or several depressions and later a deep pit (FIGURE 1*c*), it adds an oviduct segment to the pronephric kidney which by that time, has attained full size, for example, in salamanders, or has already begun to regress, as in frogs. In salamanders the blastemic tip of the funnel becomes imbedded in the wall of the primary nephric duct (FIGURE 1*d*). The entire oviduct forms as a product of proliferation and downgrowth from the blastemic rudiment (FIGURE 1*e*). In frogs, where oviduct formation starts only at the climax of metamorphosis—stages 29 to 32—union with the nephric duct never occurs, but the downward growth generally parallels at some distance the mesonephric duct (FIGURE 2). This same pattern of independent oviduct development is maintained also by all amniotes (FIGURE 1*f*).

The next changes are linked with the evolutionary reduction of the head kidneys of amniotes. In amphibians, even in the adult stage, the ostia of the oviducts maintain their position in the uppermost recesses of the body cavities, usually near the roots of the lungs. In bird embryos of four days' incubation—stage 25—pronephric vestiges are scattered through most of the cervical segments. The oviduct fields or primitive funnel depressions appear at this time in the lower part of the neck, at about the tenth to the thirteenth segment. While the oviducts grow, however, their funnels start sliding downward. At

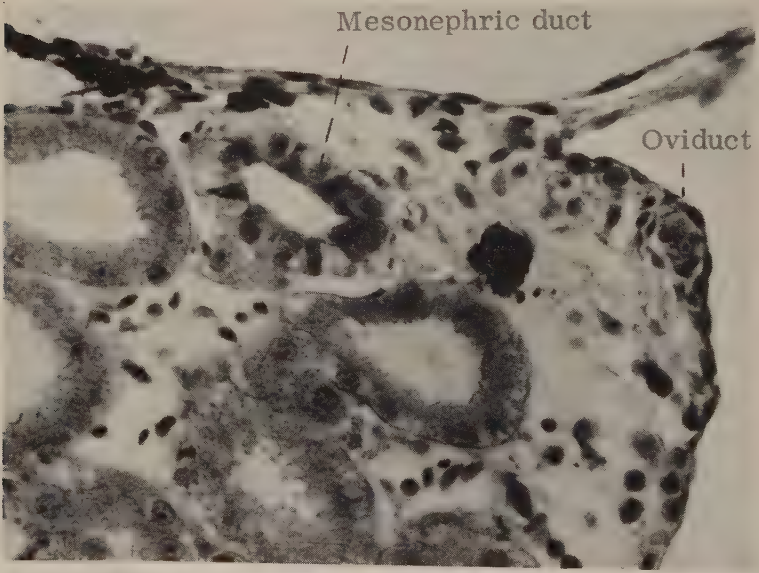


FIGURE 2. Oviduct formation in a metamorphosing female *Xenopus* (stage 32). Cross section through mesonephros at upper level of ovary shows the growing tip at some distance from the mesonephric duct. ×500.

e age of eight days—stage 34—the ostia are in the lower thoracal region.
fter this, in the male both entire ducts regress. In the female, the left ostium
scends further into the lumbar region, while the right one becomes buried in
e caudalmost pocket of the pelvis (Witschi, 1956).

In mammals, particularly in man, the pronephros is highly reduced and
agination of an oviduct funnel is delayed until stage 29. At that time, when
e human embryo measures between 12 and 14 mm., a pleuroperitoneal fold
ojects from the dorsal and lateral body wall into the celomic cavity just above
e mesonephros, the primitive gonad, and the adrenal. The thickened oviduct
ld, thrown into many heavy folds, is located in the narrow recess between
e urogenital organs and the pleuroperitoneal ligament. Because the space
very cramped, the epithelial folds spread partly on the underside of the primi-
ve diaphragm. These folds also cap the upper pole of the gonad (FIGURE
). This situation favors the simultaneous formation of several funnels. Al-
ough one usually dominates and inhibits further development of accessory
icts, double funnels and ostia are not too rarely observed variants. More-
er, the frequent, if not regularly occurring hydatid cysts and appendages of
ıman testes, epididymides, and oviducts are remnants of abortive funnels.
ıe intimate fusion of the funnel field with the upper end of the gonad is of
ırticular interest. Even after the descent of the newly differentiated ovary to
e edge of the pelvis, the funnel epithelium extends to the tip of the ovary
mbria ovarica); some folds penetrate into deep grooves of the gonad surface.
is highly probable that such ingrowths, separating themselves from the
nnel, become the source of ovarial endometriosis, one of the most common
necologic pathologies. At the reproductive age the histology of these endo-
etrial cysts and islands may be nearly identical with that of uterine endome-
ium (FIGURE 3). Functionally these bodies participate in the menstrual cycles,
th monthly discharge of blood and tissue fragments, and are known as
ocolate cysts. Neither the theory of implantation of "regurgitated" uterine
ucosa fragments (Sampson, 1921), nor Novak's suggestion (1922, 1952) that
ıder adequate hormonal stimulation the entire pelvic surface epithelia might
capable of endometrial differentiation, provides a satisfactory explanation
the etiology. Oviduct formation has never been seen to start from any
rt of the celomic lining other than that of the nephrostome or funnel fields.
indicated above, these are areas of a particular nature that belong embryo-
gically to the nephric rather than the peritoneal system. I propose the deri-
tion of endometrial teratomas from isolated fragments of funnel epithelium,
ıich may be characterized as a germinal theory; it does not exclude the possi-
lity of additional causes of endometriosis, for instance, the implantation of
ose pieces of uterine mucosa, rare as such occurrences seem to be.

Development of the uterovaginal rudiment. There can be no more real need
double sex organs, gonads and gonaducts, than there is, for example, for a
uble intestinal tract. Duplicity, practically complete in marsupials, ob-
ously evolves as a consequence of constructive principles on the basis of the
evailing bilateralism of vertebrate bodies. A tendency toward simplifica-
n is widespread. Most female birds have only a single functional ovary

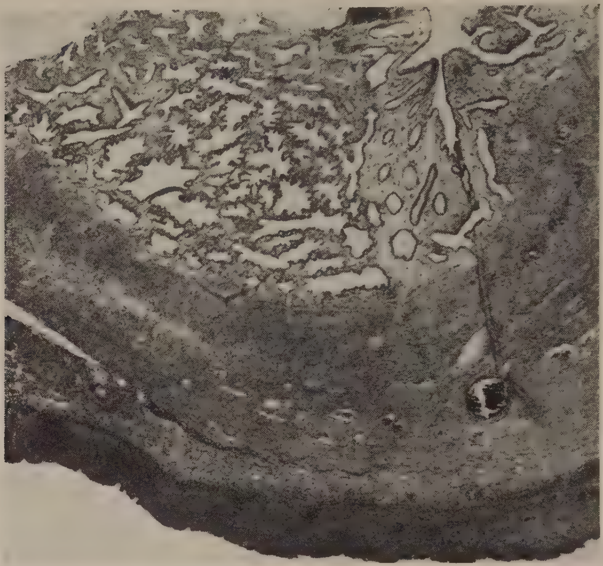
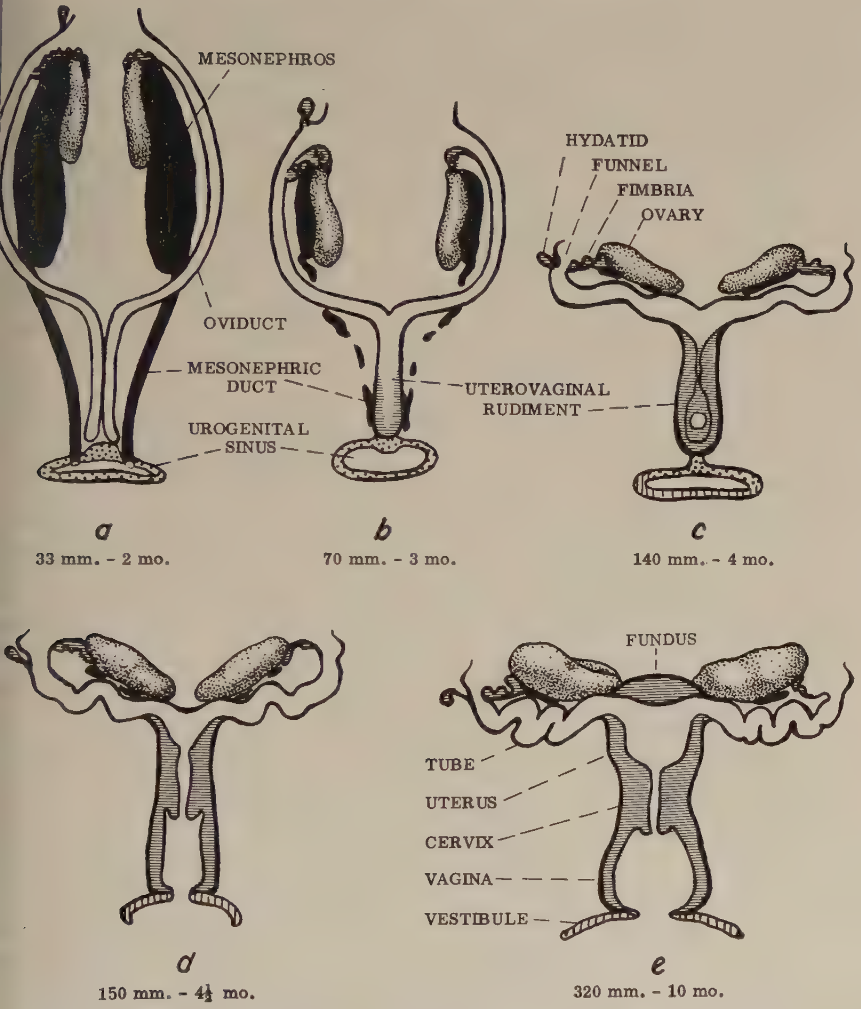


FIGURE 3. Ovarian endometriosis; fragment of a cyst showing the endometrial type of histology, particularly the development of uterine glands; ×20. (Preparation, courtesy of Edith L. Potter, Lying-In Hospital, Chicago, Ill.).

and a single functional oviduct. In some sharks the two ovaries and the ostia of left and right oviducts join in the mid-plane. Very often fusion occurs between the lower oviducts where they approach the cloaca. Even some amphibians thus acquire a single stem for otherwise separate uteri. Extensive simplification takes place in most mammals. In human embryos about two months old the tips of the oviducts have reached the lower ends of the mesonephric bodies—23 mm., stage 33. In the next few days they swing toward the mid-plane; left and right ducts meet and fuse while their tips approach the dorsal wall of the urogenital sinus—33 mm., stage 34, as shown in FIGURE 4*a*. However, even before the completion of the single uterovaginal canal sex differentiation intervenes. By the conclusion of the third month the mesonephric ducts of the female have almost disappeared (FIGURE 4*b*). In the male, the uterovaginal rudiment disintegrates, except for a small vesicular remnant attached to the sinus. This is known variously as the prostatic utricle or the male uterus, although in fact it is a vestigial vagina.

As its name indicates, the female uterovaginal rudiment typically differentiates into uterus and vagina, which are connected by a cervix (FIGURE 4*c*, *d*, and *e*). However, this is not the same in all mammals. Besides man, only apes, monkeys, some bats, and the armadillo have a simple uterus (FIGURE 5). In the rat and other rodents, almost the entire unpaired rudiment is used up in the formation of the vagina; in other words, in all animals having two uteri at the adult stage, only the prospective vaginal segments of the oviducts fuse (FIGURE 5*a* and *b*). In horses the lower parts of the prospective uteri also participate



33 mm. - 2 mo.

70 mm. - 3 mo.

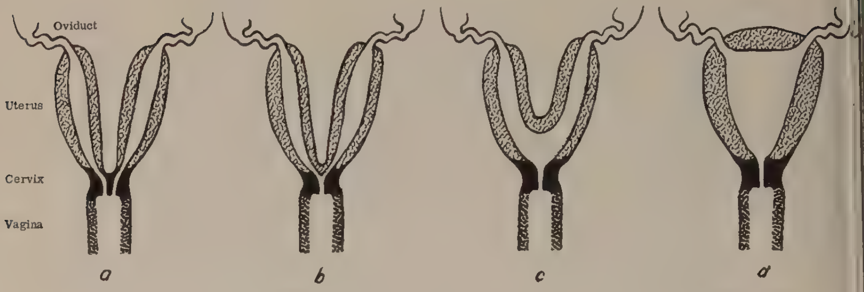
140 mm. - 4 mo.

150 mm. - 4½ mo.

320 mm. - 10 mo.

FIGURE 4. Differentiation of the gonaducts in human female fetuses. The white areas ith solid parts horizontally hatched represent the oviducts; the black, the mesonephros and esonephric ducts; the finely stippled, the ovaries; the coarsely stippled the endoderm; and e vertically hatched, the ectoderm.

the fusion (FIGURE 5*c*). Consequently the adult uterus is a single stem with ft and right horns. Pregnancy usually starts in one horn, but as the "colt c," or chorion, grows larger, it pushes through the stem into the opposite orn as well. Double uteri retain the general character of primitive oviducts, ut are distinguished by their adaptation to gestational functions. Otherwise ey resemble the tubes, that is, the least changed uppermost parts of the oviucts.



UTERUS DUPLEX UTERUS BIPARTITUS UTERUS BICORNIS UTERUS SIMPLEX

FIGURE 5. Types of mammalian uteri.

Incomplete fusion of the primitive oviducts early in the third month result in malformations of human uteri, which simulate all the types that are norma for lower mammalian groups. Even a case of functional resemblance to th equine bicornate type of pregnancy is reported in the extensive review of suc abnormalities by Jarcho (1946).

Differentiation of the urogenital sinus and uterovaginal complex. When the oviducts first reach the sinus, the point of contact lies between and slightly above the entrances of the mesonephric ducts. In male development this becomes the prostatic region. In female rabbits this attachment high on the urogenital canal never changes, as illustrated particularly well by the case of the cottontail, a species with persisting prostates in the female (Elchlepp 1952). In other species the uterovaginal rudiment gradually slides down along the dorsal wall of the urogenital sinus. In cats, horses, cattle, and armadillos (FIGURE 6) it achieves its final attachment somewhere near the middle of the original sinus. Consequently, in such species the copulation tract is composed of sinusal and vaginal sections of various lengths (FIGURE 6). In the human the uterovaginal cord finally separates from the sinus, which then is reduced to a urethra, and the attachment shifts to the ectodermal vestibule. The higher rodents—the hamster, rat, and guinea pig—attain an even more complete separation of external orifices for urethra and vagina, the introitus of the latter appearing in the perineum, which is shown in FIGURE 14 (Bruner and Witschi, 1946).

Incomplete descent, with or without the opening of the vagina into the urogenital canal, is a relatively frequent malformation in the human female. Because of the now recognized hormonal etiology, its various manifestations are discussed in the experimental section of this paper.

The actual opening of the oviducts into the cloaca occurs in amphibians and birds only at the approach of the first egg-laying season. No systematic observations have been made of amphibians, but casual examination of serial sections of this region in immature frogs and salamanders have always shown intact terminal epitheliums. The administration of high doses of testosterone to larval salamanders causes these to open into the cloaca. From this observation Mintz (1947) concludes that the normal perforation at maturity is brought out by an increased output of ovarian hormones. Greenwood (1935), who ade a careful study of 169 hens, found that perforation occurred when they ere between 140 and 284 days old. In a similar flock, the first egg was laid hen they were between 149 and 310 days old. Parallel examinations of the varies, the oviducts, and the combs led to the conclusion that perforation is a ormonally controlled process, and not a forced rupture due to egg-laying.

In mammals, with their more highly differentiated oviduct systems, perforaon usually occurs relatively earlier. In the hymenal membrane of female uman fetuses an opening usually forms some time during the second half of trauterine development. However, in rare instances the membrane remains tact until birth or adolescence, when it becomes an obstacle to menstrual ischarge. In rats, mice, and the guinea pig, the vaginal opening is definitely

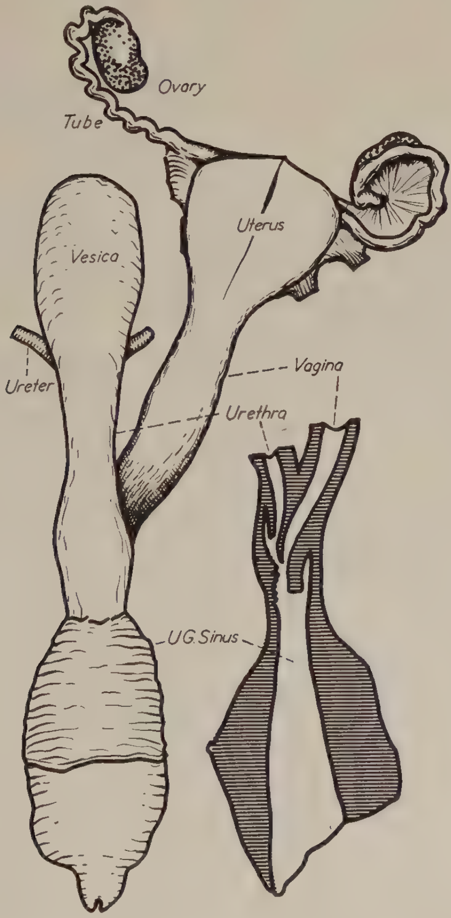


FIGURE 6. Female sex organs of the armadillo. At the right, a sagittal section shows e confluence of the ureter and vagina into the urogenital sinus.

under estrogenic control. In the hamster, however, perforation is independen of hormonal stimulation, and occurs eight to nine days after birth, in bot castrate and intact females.

For many years lengthy discussions have been carried on about the origi and nature of the epithelium that lines the vagina and the uterus. Histologically it resembles the ectodermal epidermis during estrous cornification stages but it also approximates an endodermal intestinal epithelium under gestational anestrus, or castrate conditions. The extreme divergence of opinion among experts may be illustrated by the following quotations: "It is now known that the *endodermal* epithelium of the urogenital sinus invades this level of the genital cord and replaces the Müllerian epithelium wholly or in part," (Arey 1954). Burns (1942*a*), resting his case partly on some work by Raynaud asserts, ". . . this origin has been traced to the *ectodermal* part of the primitive cloaca." Exhausting all possibilities by holding steadfastly to the conventional view of the mesodermal nature of the vaginal epithelium, Lippmann (1940) declares: "Die Vagina entwickelt sich ausschliesslich aus den Mül-

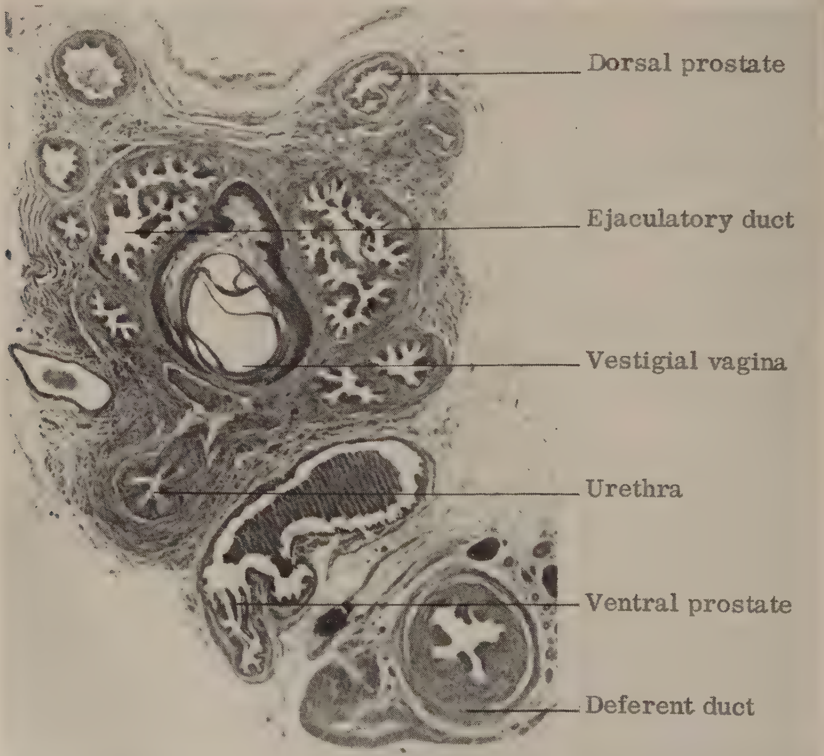


FIGURE 7. Cornified vaginal epithelium in the vestigial vagina of a male hamster after estradiol stimulation (from Stolpe, Thesis, 1948); ×17.

erschen Gängen."* Certain observations on the development of the hamster peak decidedly for the mesodermal nature of the vaginal epithelium, and gainst any secondary replacement by invasion. In the hamster, as well as in nan, the vestigial male vagina neither opens nor descends, but forms a cyst hat maintains its place at the dorsal border of the prostatic, or endodermal, urethra between the ejaculatory ducts. If estrogen treatments are adminis-ered to newborn male hamsters for one week, the lining epithelium of this vaginal cyst acquires the stratified squamous and cornified character that is ypical for the female estrous vagina (FIGURE 7). By no stretch of the imagination could one assume that ectodermal cells might have ascended to this evel. Although an endodermal cushion or sinovaginal cord establishes early he link between sinus and uterovaginal rudiment, it remains histologically listinct and does not respond by cornification to estrogens (FIGURE 16).

Experimental Analysis

Extirpation of the oviduct field. When uniting tailbud embryos of salamanlers in parabiosis, one usually removes from the graft area, which includes the ronephric bulge, only surface ectoderm. However, if the deeper pronephric udiment is also excised, no oviduct is formed on this side of the particular nimal. More exactly, after removal of the oviduct field, neither the funnel or the lining tube epithelium develops; nevertheless, the celomic epithelium ises into a vestigial fold running alongside the mesonephric duct (FIGURE 8). This illustrates the fact that the epithelium that lines the lumen of the oviducts nd uteri and forms the uterine glands is derived by downgrowth from the rimordial pronephric rudiment, while the mesenchymal parts of the oviducts nd of the uterine endometria, as well as all of the muscle layers and ligaments, re added *in loco*, partly under the organizing influence of the epithelial inner ube. It is noteworthy that after such extirpations no restitution occurs, ven if the operations are performed long before the oviduct fields become isibly organized (Riley, 1934). Evidently preprimordial determination of he oviduct immediately follows that of the pronephric funnels, and this occurs o later than during the neurula stages.

Suppression of oviduct formation by testosterone. If salamander larvae are aised in weak solutions of testosterone before and during the stages when viduct development normally proceeds, neither funnels nor tubes ever form Foote, 1941). The inhibitory capacity of methyltestosterone was found to e absolute in a wide range of dilutions—between 0.01 and 500 μg. per liter of quarium water, (Bruner and Witschi, 1954). If hormone administrations re started late, the downgrowth is stopped at whatever level it had reached FIGURE 9). However, the oviducts become enlarged and develop thick mucous pithelia, as they normally occur in females approaching the breeding season Mintz, 1947). Similar reactions follow the administration of estradiol or ther gynogens, except that the initial formation of a short and wide funnel is ot suppressed even by very early administration of high dosages.

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* The vagina develops entirely from the Müllerian ducts.

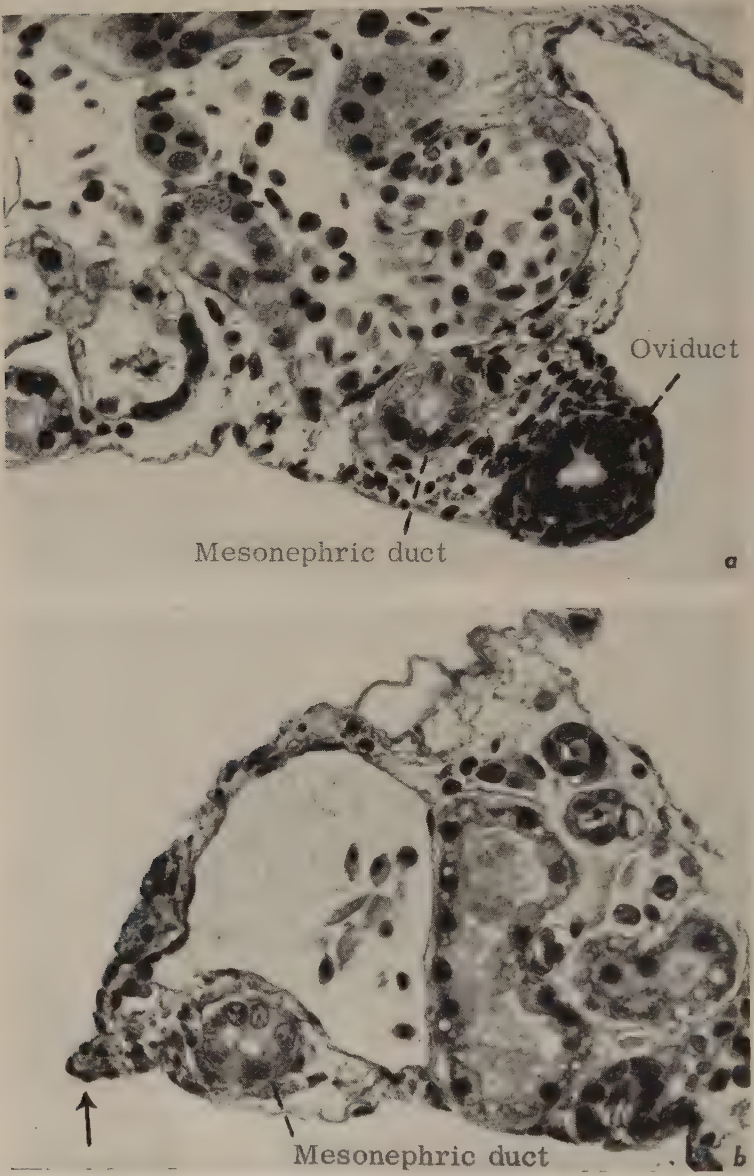

FIGURE 8. Cross sections through mesonephric bodies of a 17½-month-old *Ambystoma tigrinum*. The right oviduct field was removed at the tailbud stage. (*a*) The edge of the right mesonephros with normal oviduct; (*b*) slight peritoneal fold (arrow) along the right mesonephros, without oviduct tube. ×250.

These experiments confirm the general impression gained from purely descriptive studies, namely, that oviducts form by self-differentiation from specific preprimordia of the pronephric funnel areas. Even though, in amphibians as in birds and mammals, oviduct formation normally starts during or after sexual differentiation of the gonads, it proceeds entirely independently of the latter. Sex hormone administrations neither produce supernumerary funnels nor promote the downgrowth of the tubes. In fact, testosterone abolishes funnel

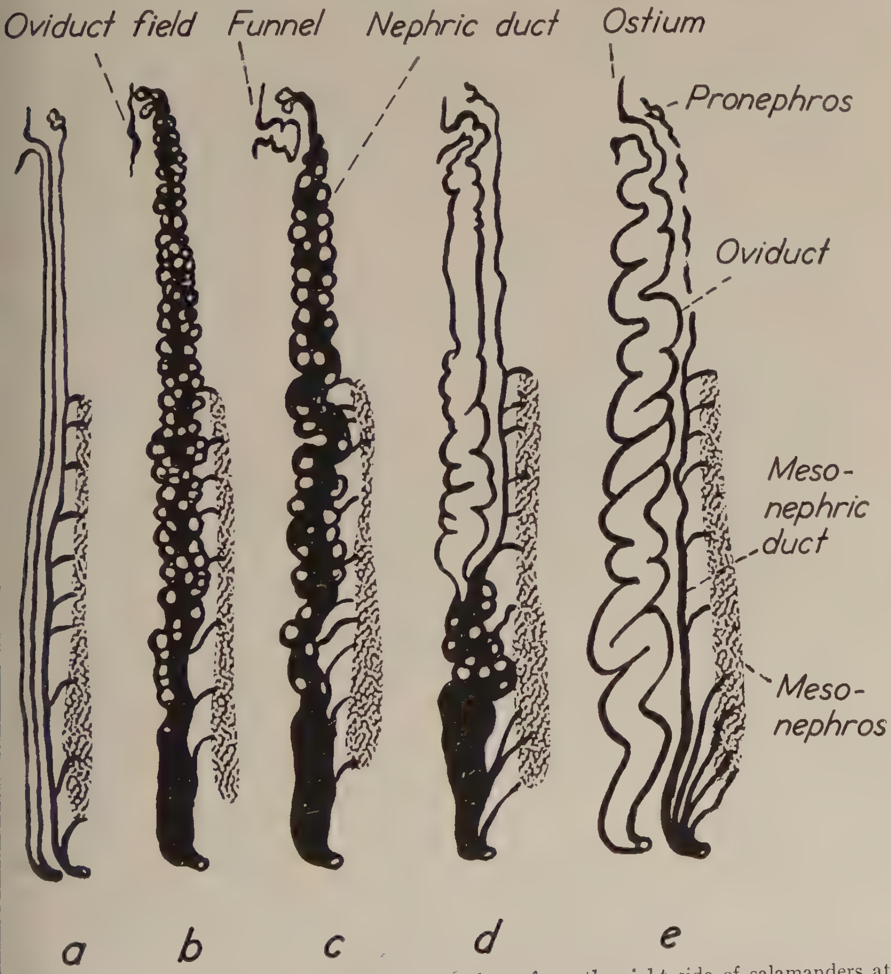

FIGURE 9. Simplified drawings of the gonaducts from the right side of salamanders at about the metamorphosis stage: (*a*) control with oviduct and nephric duct, the pronephros regressing; (*b-e*) effects of testosterone injections; (*b*) injections starting before stage of oviduct formation—no oviduct, entire nephric duct highly stimulated; (*c*) and (*d*) injections starting during progress of oviduct formation—downgrowth stopped, oviduct rudiment and undifferentiated part of nephric duct highly stimulated; (*e*) injections started after completion of oviduct formation—oviduct highly stimulated.

formation and both androgens and estrogens stop the downgrowth of tubes. In either case the hormones efface developmental patterns and capacities of blastemic areas. I proposed the term "adultization" (Stoll, 1950) for this process, which obliterates the embryonic character of the cells composing funnel fields or tips of growing tubes. However, it is significant that testosterone does not destroy the latent competence of oviduct fields that are still unactivated. Administration of high, often sex-reversing dosages to newly hatched larvae or to older ones that already are passing through the phase of gonadal differentiation leaves the oviduct-forming capacity intact. Normal oviduct formation follows if the treatments are discontinued at the proper age and stage. Therefore, the canceling effect must be due to interference of the hormone with the activation of the morphogenic process.

The combination of partial or complete absence of uterus and tubes with the presence of normal ovaries is a rare but well-known class of gynecologic abnormalities. Although the etiology is still unknown, the possibility of abnormal hormonal conditions at the stage of the 13 mm. human embryo—stage 29—might be given some consideration.

The experiments with *Ambystoma* are of further interest because they bring into focus an intimate relationship between the tip of the oviduct and the wall of the mesonephric duct that is characteristic and probably unique for salamanders. No matter where its downgrowth is stopped, the oviduct does not end bindly; instead, it opens into the mesonephric duct. This is very different from results of corresponding experiments with frogs and birds, in which, according to expectations, similarly arrested oviducts end without any opening at some distance from the mesonephric ducts (Segal, 1951; Stoll, 1950).

Functional stimulation of gonaducts by steroid hormones. Having taken shape, the oviducts of salamanders are immediately sensitive to androgens as well as to gynogens, reacting by enlargement and mucification. A similar responsiveness to androgens is also characteristic for birds, while both groups of animals remain unaffected by progesterone administrations (Witschi, in preparation). The question of whether progesterone plays any role in the oviduct physiology of nonmammalian vertebrates is still open. The meaning of the responsiveness of oviducts to androgens and its possible relationship to the evolution of the progestational phenomena in mammals remains obscure. Even in the opossum the oviducts of pouch young are partially stimulated by androgens (Burns, 1942*b*).

The salamander experiments also reveal dramatic changes in responsiveness of the developing nephric ducts to testosterone administered at various stages. The hormone produces no effect in larvae up to the fifth month, which is the normal time for oviduct formation and regression of the pronephric duct segment to begin. At this time the nephric duct in its full length becomes responsive, even excessively so, while oviduct formation is simultaneously suppressed (FIGURE 9*b*). However, if administrations are delayed, it is the oviduct that becomes hypertrophic, while the accompanying pronephric and mesonephric duct segments show only a moderate stimulation (FIGURE 9*c* and *d*). It is remarkable that the nephric duct below the entrance of the oviduct still esponds as strongly as the oviduct. Seemingly, as the tip of the oviduct ides along the primary nephric duct, the capacity for excessive response, nd particularly for mucification, passes from the nephric duct into the oviuct. The reaction pattern of excessively stimulated nephric ducts, including he histological transformation of their tube epithelia, is similar to that of viducts. Nevertheless, the nephric ducts respond only to androgenic stimuation, whereas the oviducts react to both classes of sex hormones.

Duct differentiation in relation to gonadal sex. Sooner or later, following esticular and ovarian differentiation, the development of the gonaducts also ssumes a male or a female pattern. One of the primitive duct systems coninues growing and differentiating, while the other remains stationary or reresses and, in some instances, completely disappears. In female birds and in nale leopard frogs (*Rana pipiens*) oviducts and deferent seminal ducts both ersist, usually in full length, to the adult state. However, even in these nstances the relative prevalence of the system that conforms with the gonadal ex is recognizable.

The factors that bring about the concordance of differentiation of ducts and ex glands are still incompletely known, but much progress in their elucidation as been made in the last ten years through the work of two French investigaors. Jost (1947) removed the gonads of rabbit fetuses by surgery before the tart of duct differentiation, and Raynaud and Frilley (1947) castrated mice t similarly early stages by means of X rays; in both experiments it was observed hat in all gonadless animals the mesonephric ducts disappeared, while the viducts continued developing and differentiating in typically female fashion. t is obvious that the female type develops by self-differentiation, the male type nder the inductive guidance of testes. This generalization is supported also y the now well-known fact that human individuals with congenital agenesis of he sex glands have a completely female gonaduct system, even those who, ccording to the sex chromatin test, are of male genetic sex. Irrespective of enetic sex, in the absence of gonadal influences the uterovaginal rudiment ifferentiates into uterus, cervix, and vagina. The tip of the latter grows in ength and descends to the vestibular position, separating completely from the rethra.

In an interesting experiment Jost (1947, 1953) implanted a testis next to one vary of a 20-day-old female rabbit fetus. The condition attained eight days ater (FIGURE 10) indicates two inductive effects radiating from the graft, the ne maintaining and stimulating the development of the mesonephric duct and he other inhibiting and obliterating the oviduct. The diffusing agent has ften been suspected to be testosterone, released in small quantities by the etal testes. Although it is true that early injections of testosterone into pregant rats (Greene, 1942) and, to a lesser degree, also into hamsters (Bruner and Vitschi, 1946) tend to maintain the mesonephric ducts, they fail to cause egression of the oviducts. Similar results were obtained by Price (1957) with xplanted reproductive tracts of 17-day-old rat fetuses. Experimental evience that the fetal mesonephric ducts are responsive to stimulation by relaively high concentrations of testosterone cannot prove that the active agent

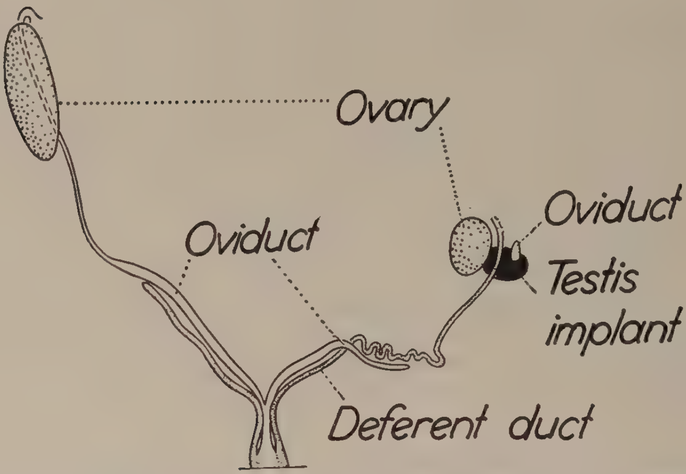

FIGURE 10. Genital tract of a 28-day-old rabbit fetus in which, on the twentieth day, a testis was implanted next to the left ovary (after Jost, 1947).

of the fetal testis is identical with an androgenic steroid. As far as the reduction of the oviducts is concerned, no information beyond the fact that the inducing agent spreads from the testis is available. Indeed, the relationship between testis and duct differentiation is more of the character of other embryonic inductions than of a typical hormone reaction.

Conditions like those produced in Jost's implantation experiment are observed also in cases of spontaneous true hermaphroditism, especially in those of the asymmetrical type. The detail of the large series of lateral hermaphrodites in mice (Hollander *et al.*, 1956) and in man (Grumbach *et al.*, 1955) reveals again that the stimulation of mesonephric ducts as well as the suppression of oviducts spread from the testes and to some degree from ovotestes. The fact that sometimes the effect is more extensive in one than in the other system suggests the separate existence of oviduct-repressing and mesonephric duct-stimulating principles. However, the differences may possibly result from timing conditions.

Although sometimes even very small and atypical testes produce masculinization of the duct system, in the case of male pseudohermaphrodites apparently normal testes fail partly or completely to induce the expected coordinations. Male individuals—according to sex chromatin tests—may develop a complete and fully differentiated female gonaduct system. In extreme cases the presence of testes interferes as little with the self-differentiation of tubes, uterus, vagina, and the reduction of mesonephric ducts as does the absence of gonads in the above-described agenesis cases (Wilkins, 1957). More often, of course, the insufficiency of the testes is not absolute and sometimes may change quantitatively and periodically. Considerable variation in the progress of masculinization of the ducts and of the phallic rudiment is characteristic for human fetuses ranging in size from 25 to 60 mm. (Witschi, 1957). Ordinarily by the

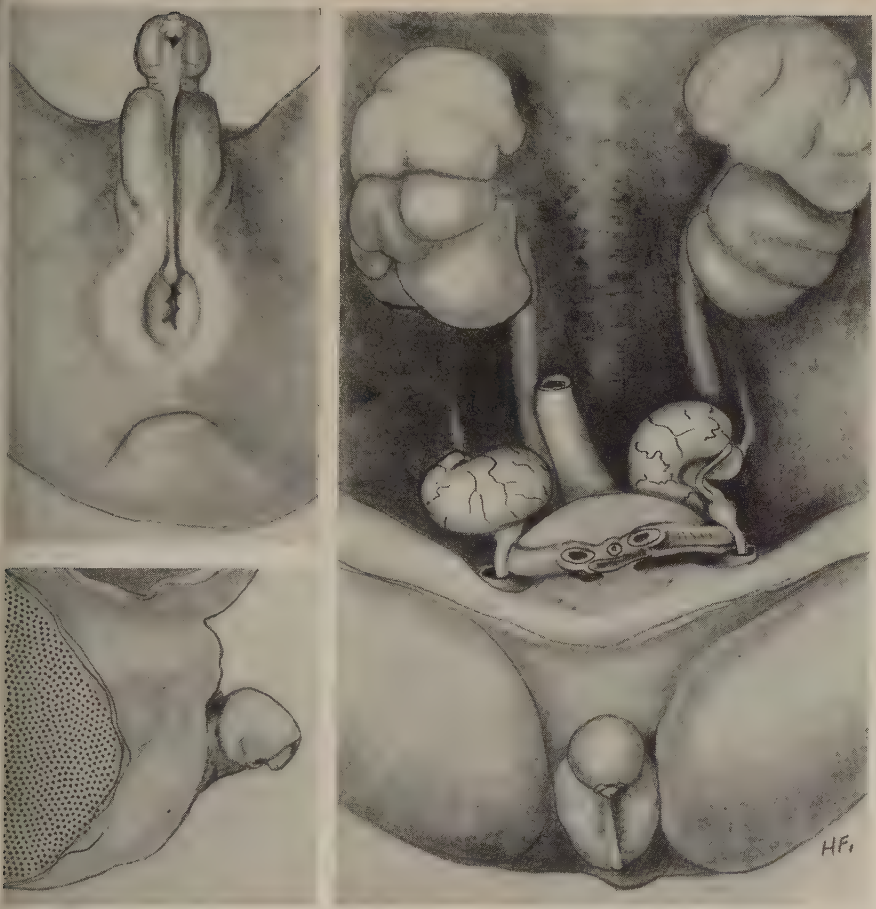

FIGURE 11. Genital system of normal male human fetus of 60 mm., crown-rump length.

latter stage (FIGURE 11) the testes have descended to an inguinal position and the raphe of the penis is complete, but it is not unusual to find fetuses (FIGURE 12) with undescended testes, a genital ligament containing some remnants of oviducts and uterovaginal canal, and a phallus of clitoridean type. It is possible that the second case represents the fetal form of the first degree of male pseudohermaphrodism, usually recognized as hypospadias. The genetics and etiology of this class of inter-sexuality have been discussed elsewhere (Witschi *et al.*, 1957).

Modification of urogenital sinus and vaginal development under the influence of steroid hormones. The golden hamster is a favorable animal for a study of the final phase of duct development because, with a short gestation period of only sixteen days, the young are born at a very precocious stage. At birth the

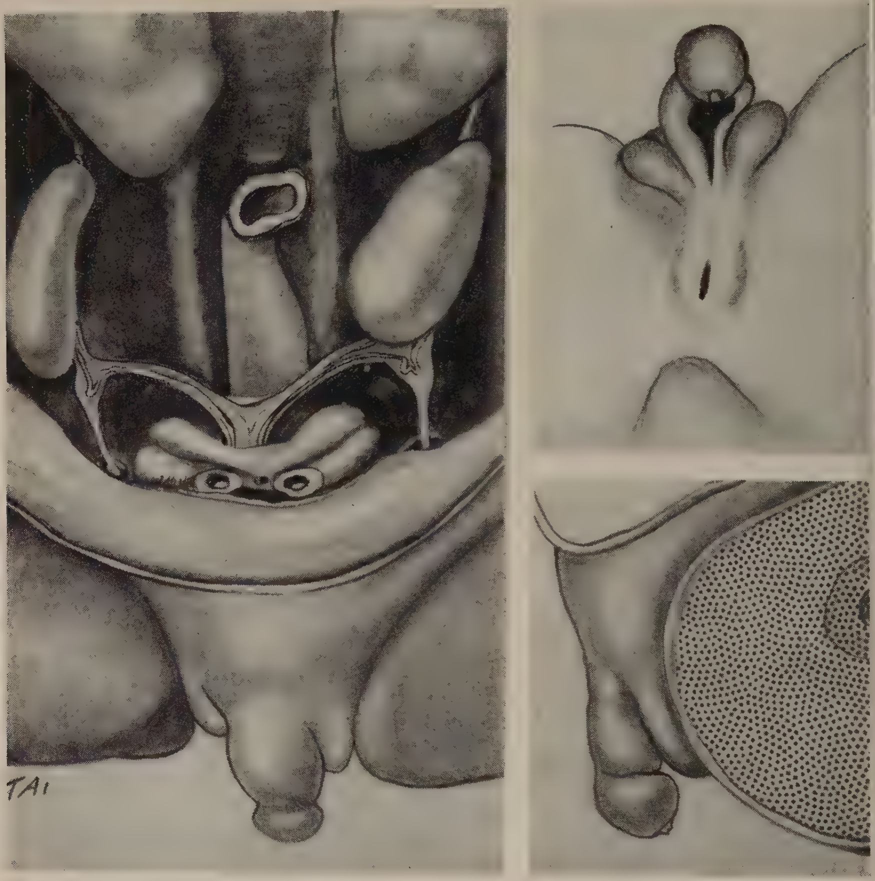

FIGURE 12. Genital system of male human fetus of 56 mm. with retarded descent of testes and incompletely masculinized secondary sex characters.

uterovaginal rudiment is still an undifferentiated canal that has barely started to slide down along the dorsal wall of the urogenital sinus (FIGURE 13). At this time it is attached at the boundary of the prostatic and glandular portions of the prospective urethra. Within the following week the descent of the junction is completed. On approaching the vestibule, the mesodermal wall of the rudiment makes a new, direct contact with an ectodermal invagination from the perineal depression—the latter being the dorsal remnant of the vestibule after separation and closure of a clitoridean urethra by a raphe (FIGURE 14). At the end of the eighth day this ectodermal introitus breaks through into the lumen of the vagina. During the first postnatal week the uterovaginal rudiment differentiates into a long vagina and a cervix, with separate canals leading into the paired uteri (FIGURE 14). Injection of androgenic or gynogenic

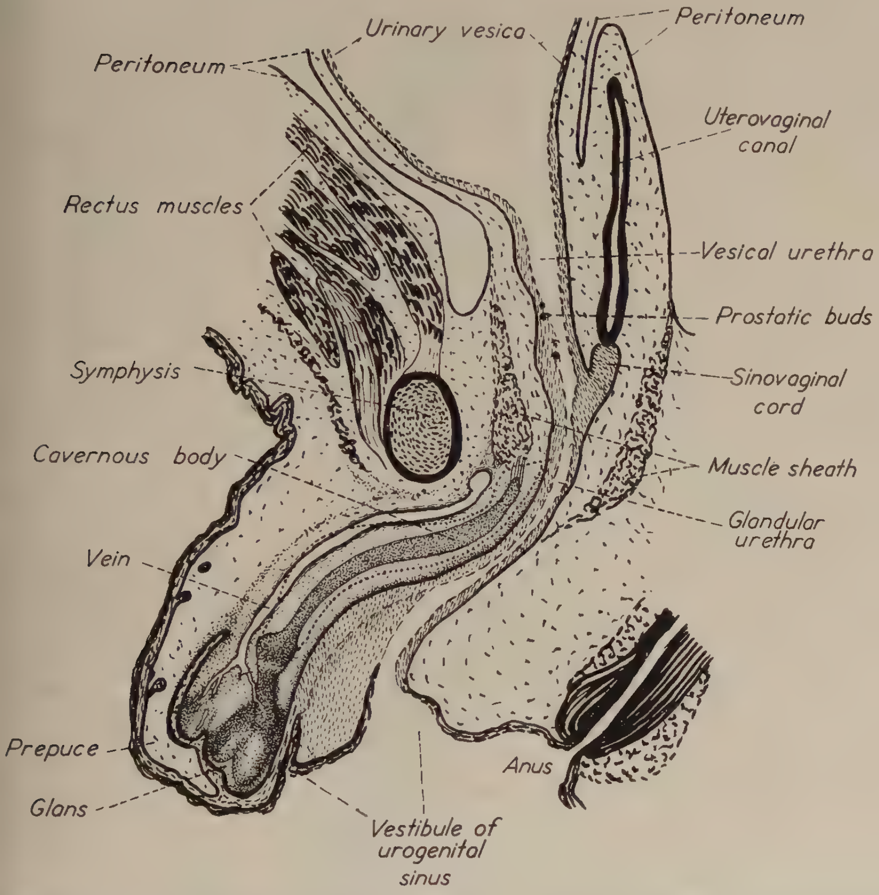

FIGURE 13. Newborn female hamster. Sagittal section through pelvic organs (Bruner and Witschi, 1946). ×33.

steroid hormones stops the descent almost instantaneously (Bruner and Witschi, 1946). If testosterone is administered in sufficient quantity (6 mg.) to the mother any time after the ninth day of pregnancy, the attachment remains located in the upper prostate region. However, if injections are started only at birth, the relatively short vagina connects with the urethra between its glandular and cavernous portions (FIGURE 15). There is no interference with the differentiation into cervix and uteri. However, along with the downward movement the growth in the length of the vagina is also checked. Here again we meet with the phenomenon of adultization, that is, precocious disappearance of embryonic blastemas. In addition, androgen injections induce the development of prostates, urethral and bulbo-urethral glands, cavernous bodies, and a considerable phallic growth, but at the same time they cause

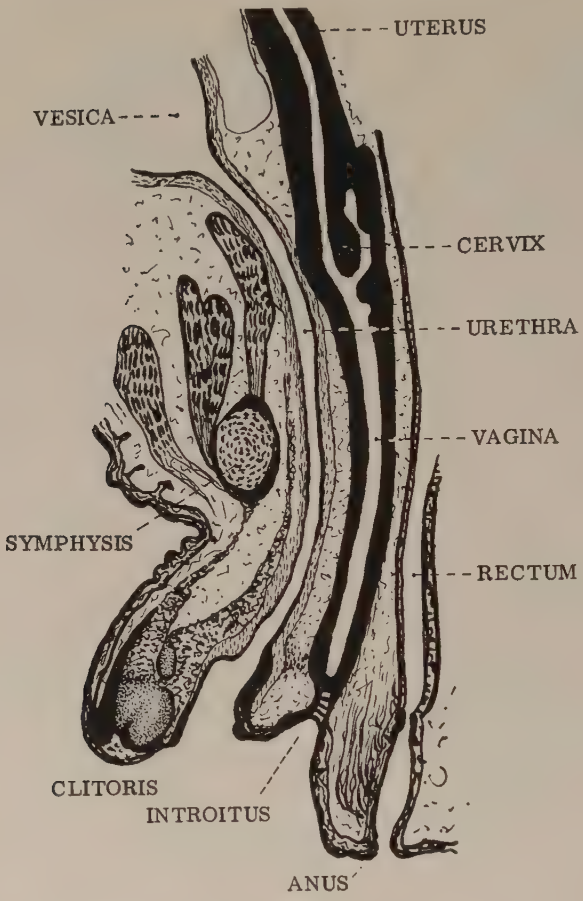

Figure 14. Sagittal section through pelvic organs of a week-old female hamster (postpartum). ×15.

hypospadia (figure 15). As one might expect, the injection of estradiol does not produce the masculinization features, but it does stop the downgrowth of the tip of the vagina and hypospadia (Stolpe, 1948). By the end of the week the vaginal epithelium is cornified. The still relatively short organ opens through the degenerating endodermal sinovaginal cord into a diverticulum, possibly ectodermal, that grows up from the lower urethra in a manner somewhat reminiscent of the normal perineal introitus (figure 16). If junction is made at a higher level, no similar ingrowth seems to occur.

It is obvious that the failure of the vagina to reach its normal length and to separate from the urethra cannot be considered as masculinization. Androgens as well as estrogens act as inhibitors, exhausting essential stores of blastemic cells. Reports of other instances of the teratogenic action of steroids that can

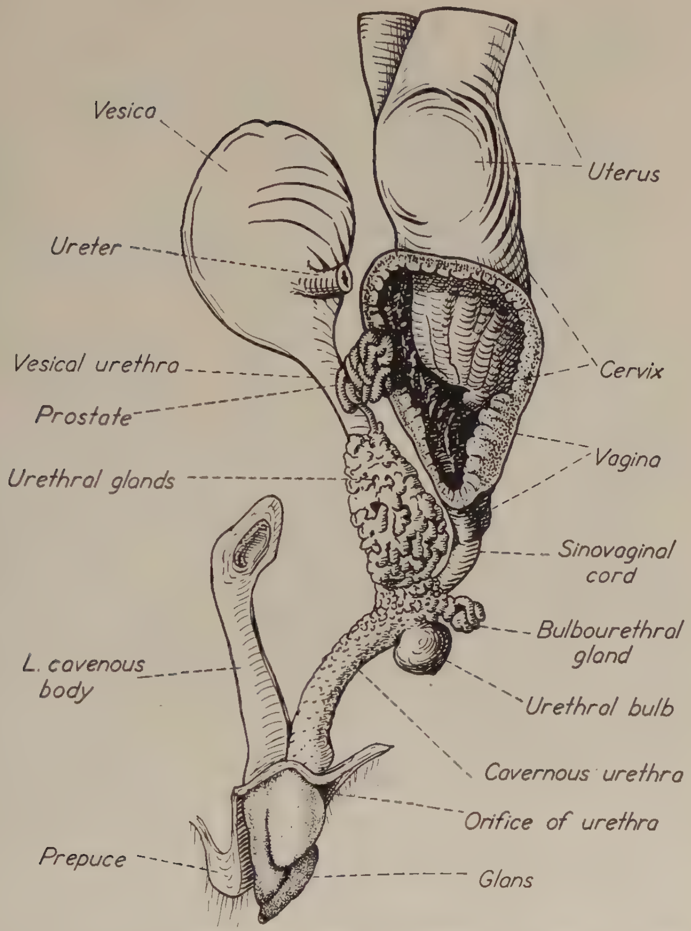

FIGURE 15. Pelvic and external sex organs of 12-weeks-old female hamster injected daily during three postnatal weeks with 1 mg. testosterone (Bruner and Witschi, 1946). ×5.5.

be understood on the same principle of adultization are rapidly increasing in number.

The final condition attained by steroid-treated females resembles the normal relationship of vagina and urethra in such animals as the rabbit and the armadillo (compare FIGURES 15 and 16 with FIGURE 6). It appears from the descriptions of Wells and van Wagenen (1954) that injecting pregnant monkeys with testosterone produces a similar series of modifications in the female offspring as those described above for the hamster.

The androgen injections duplicate in almost every respect the effects of adrenal hyperplasia in the so-called adrenogenital syndrome of human female pseudohermaphrodites. In both instances masculinization appears first,

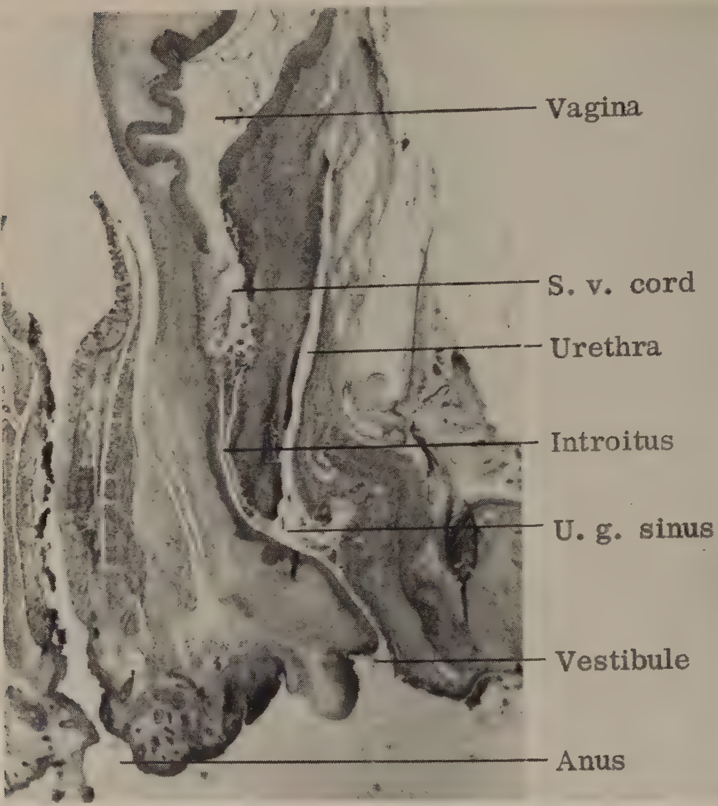

FIGURE 16. One-week-old female hamster injected daily after birth with 50 μg. estradiol. The endodermal sinovaginal cord, which is labeled S. v. cord, is degenerating (Stolpe, 1948). ×25.

that is, in the lightest cases, in the phallic and vestibular region. The gradient of response ascends from the external genitalia toward the sex glands. This direction is exactly opposite to that taken by the masculinizing induction that issues from the gonads in normal male differentiation, in the cases of male pseudohermaphrodism (FIGURE 17), or in the cattle freemartins. This rule is not invalidated by the fact that the testes of male pseudohermaphrodites, and of pseudomales, at later stages usually release varying amounts of androgens and gynogens that secondarily may modify and complicate the aspects of intersexuality in these classes of sex abnormalities.

Recently, cases of female pseudohermaphrodism in newborn children, without evidence of adrenal malfunction, have been reported. In some instances the pregnant mothers had been given considerable amounts of androgenic hormones (Zander and Müller, 1953; Hoffmann *et al.*, 1955), or progesterone (Wilkins, 1957). The possibility of inadvertent virilization of the fetus and

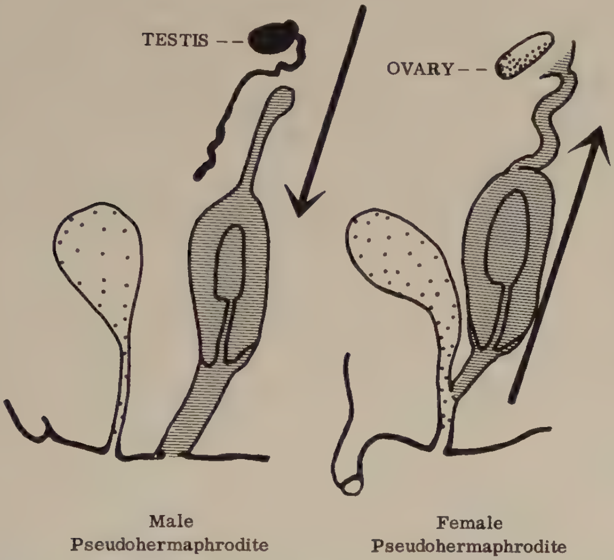

Figure 17. Gradients of diminishing effects in pseudohermaphrodism.

the production of severe malformations by steroid therapy during pregnancy certainly deserves serious attention.* The experiments with animals seem to indicate that only excessive dosages may reach a teratogenic threshold.

Summary and Conclusions

(1) By the rules of homology, the epithelial inner tube of the oviduct and its specializations such as uterine glands and vaginal epithelia are a pronephric derivative.

(2) Embryologically the tube arises by self-differentiation from a primordial oviduct field that is potentially determined and localized not later than at the tailbud stage.

(3) Facts and present embryologic concepts favor a germinal theory of endometriosis as against the view that endometrial differentiation may start from any part of the peritoneum under particular hormonal stimulation.

(4) In rodents, man, and possibly all other mammals, the anatomic differentiation of the female gonaduct system occurs without gonadal control. Inasmuch as, besides this no other inductive influences are known to enter, the prepuberal development of tubes, uteri, and vagina appears as a process of self-differentiation.

(5) The regressive changes in the oviducts that normally occur in male fetuses are induced by some agent spreading from the testes. Since it has never been

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* After this article went to press I learned of the publication of a paper by L. Wilkins, H. W. Jones, Jr., G. H. Holman, and R. S. Stempfel, Jr. Masculinization of the female fetus associated with administration of progestins during gestation: non-adrenal female pseudohermaphrodism. J. Clin. Endocrinol. and Metabolism. **18:** 559–585. June 1958.

possible to imitate this process by endocrine administrations, the inductor cannot be assumed to be of the nature of a steroid hormone.

(6) The developing oviducts are responsive to steroid administrations. The observed effects range from suppression of the oviduct-forming capacity of the primordial funnel field to inhibited growth and descent of the vaginal rudiment. Teratogenesis results from precocious exhaustion of embryonic blastemas, that is, adultization, and a consequent developmental arrest.

(7) At various stages and to variable degrees the gonaduct rudiments can also react with some functional changes such as precocious development of puberal characters.

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GROWTH AND DEVELOPMENT OF THE UTERUS: THE RELATIONSHIP OF EPITHELIUM TO MESENCHYME

By Peter Gruenwald
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If biologists ever believed that each of the three germ layers of the embryo produces either only epithelial or only mesenchymal tissues, this notion has long been dispelled. Numerous observations of transition between the two types are on record as occurring within cell aggregates derived from one germ layer. Consequently, one must not regard epithelial or mesenchymal characteristics as definitive and unalterable attributes of any cell or group of cells and its progeny. The property of epithelial or mesenchymal structure can refer only to the relationship between adjacent cells present at the time of observation, without prejudice as to past or future change.

Of all parts of the embryo, the walls of the coelomic cavity and their derivatives probably show the greatest lability with regard to epithelial versus mesenchymal structure. This is manifest in development, structure, and pathological formations of most parts of the urogenital tract, including the uterus, as well as the serous membranes themselves and the adrenal cortex.[1]

Morphologically, one finds this trait expressing itself in the serous membranes of young embryos by the absence of any separation of superficial epithelium and underlying mesenchyme. FIGURE 1*a* shows, in an example taken from a human embryo of the third week, a layer of superficial cells that appear epithelial on the side facing the serous cavity, and mesenchymal on the opposite side. On their mesenchymal side these cells appear much like the nearby cells of the deeper layers of tissue. Eventually this layer acquires the characteristics of an epithelium, and it is then sharply demarcated from the mesenchyme. However, this demarcation is not final; it fails to develop, or disappears again in areas of very active proliferation such as, for instance, in the early primordia of the gonads (FIGURE 1*b*) and the adrenal cortex (FIGURE 1*c*). It is pointless to argue whether, in these instances, the epithelium or the mesenchyme proliferates; the distinction between the two disappears and a mass of uniform cells proliferates in which, subsequently, both epithelial and mesenchymal cell groups differentiate. In the adrenal cortex the intermediate characteristics remain permanently apparent as a gradual transition from the epithelial zona glomerulosa to the nonepithelial zona fasciculata, in which each cell is enveloped by lattice fibers.[1] The present example (FIGURE 1*d*) shows this in the adrenal cortex of an adult dog.

In the case of the uterovaginal canal and its principal embryonic primordium, the Müllerian ducts, the peculiar close relationship of epithelium and mesenchyme manifests itself in a somewhat different form. The Müllerian duct arises from a portion of the coelomic epithelium which, at the time, is well demarcated from the mesenchyme and remains so while the duct grows caudad. The growing tip of the Müllerian duct is at first found within the basal membrane of the Wolffian duct;[2] in fact, the association is so close that,

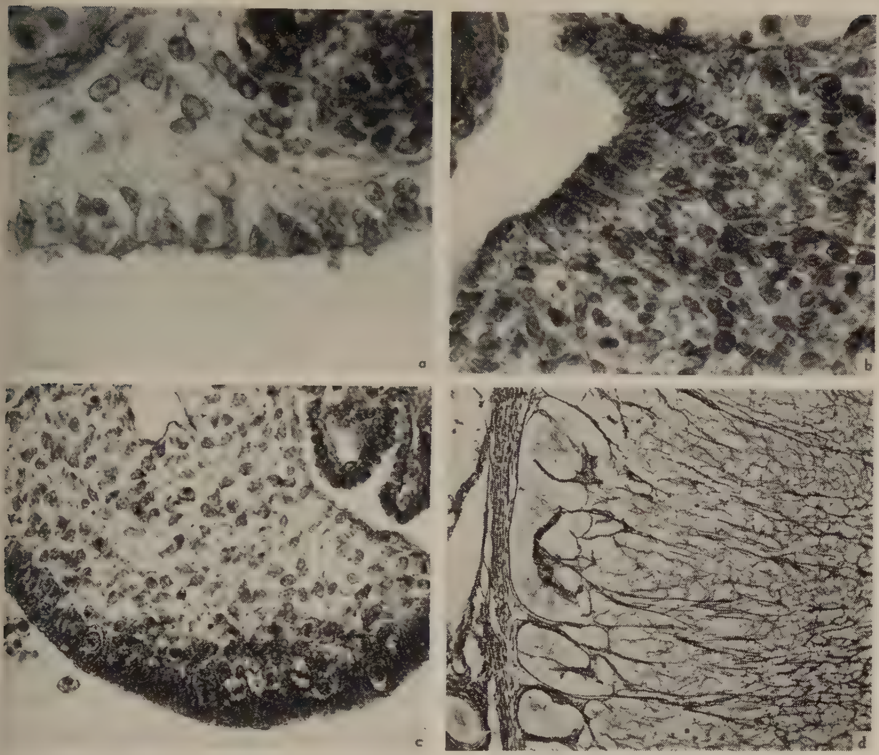

Figure 1. (*a*) Wall of the coelom of a human embryo of the third week, showing a lining intermediate between epithelium and mesenchyme. (*b*) Gonad primordium of a 8-mm. human embryo, showing no separation of epithelium and mesenchyme. (*c*) Primordium of the adrenal cortex of a 7.5-mm. human embryo, also showing the absence of distinct epithelium. (*d*) Adrenal of an adult dog, in which silver impregnation of the lattice fibers shows, in each cell cord, a gradual transition from epithelial to mesenchymal structure. Figures *a* and *c* are reproduced from originals of figures 1 and 3, Peter Gruenwald.[1]

when the Wolffian duct is interrupted experimentally or spontaneously, the Müllerian duct cannot grow past that point by itself.[3] Eventually a basal membrane develops between the two ducts. These events, as well as the subsequent processes of differentiation that will be described, progress in the caudal direction, much as does the growth of the Müllerian duct itself. It is therefore possible to see in a suitable stage all phases along one duct, with the earliest steps in the caudal portion and more advanced ones more cranially (figure 2).

Shortly after a basal membrane first appears between the Wolffian and Müllerian ducts, fine lattice fibers appear within part of the tissue of the Müllerian duct (figures 2*d*, 3*a*, and 4*a*), while other parts remain unchanged. A new basal membrane then separates the part without lattice fibers from that with such fibers (figures 2*e*, and 3). The former becomes the definitive Müllerian duct, whereas the remainder acquires more lattice fibers and merges with the surrounding mesenchyme after the basal membrane of the original Müllerian duct has disappeared as such (FIGURES *2f* and *3d*). When this change is completed, part of the mesenchyme surrounding the Müllerian duct is derived

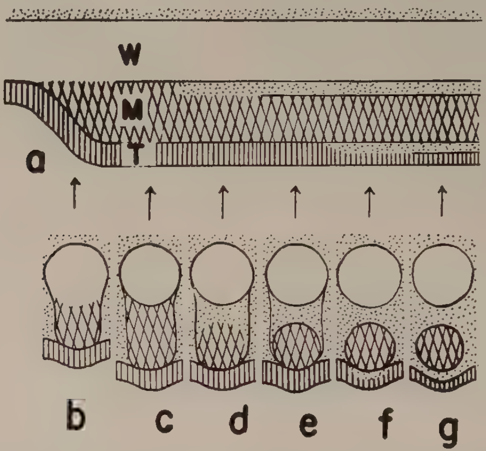

FIGURE 2. Diagram of the origin of mesenchyme from the epithelium of the Müllerian duct (M) and tubal ridge (T). In *a* the caudal part is on the left; *b* to *g* show cross sections at the levels indicated by the arrows. W is the Wolffian duct. Reproduced by permission from *Archives of Pathology*, Gruenwald, Jan., 1943.

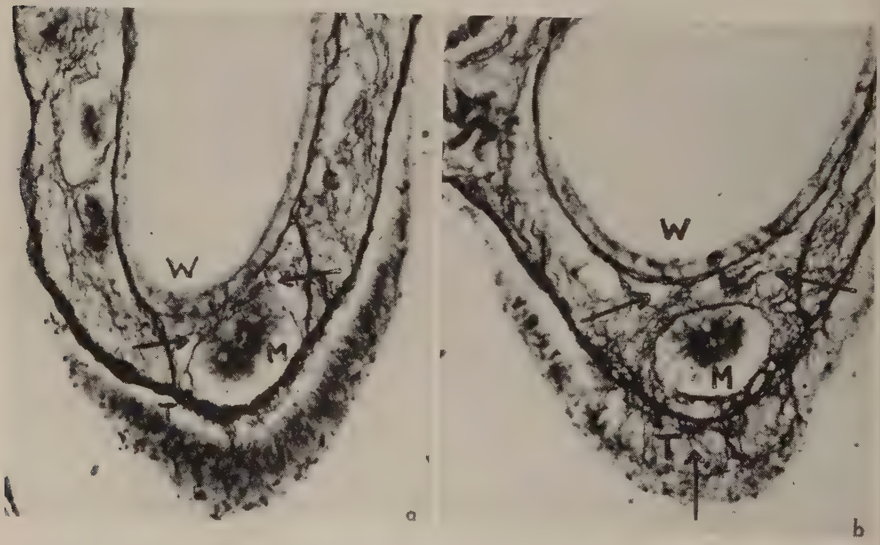

FIGURE 3. Sections of part of the urogenital ridge of a chick embryo of 6 days and 3 hours, with silver staining of the lattice fibers. M is the Müllerian duct; T, the tubal ridge (mesenchyme originating from these is indicated by arrows); W, the Wolffian duct.

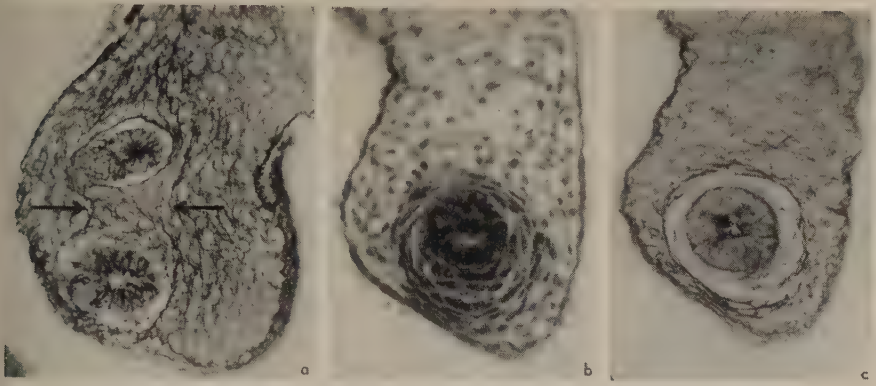

FIGURE 4. Sections from human embryos. (a) From a 23-mm. embryo, shows mesenchyme between the Wolffian and Müllerian ducts enclosed in the old basal membrane (arrow); (b) and (c) from an embryo of 67-mm. crown-heel length, show a distinct layer of fibroblasts surrounding the Müllerian duct. FIGURE *a* is reproduced by permission from *Archives of Pathology*, Gruenwald, Jan., 1943.

from cells of the duct itself; it is indistinguishable from the mesenchyme previously present in the area.[1, 4]

A similar change occurs, somewhat later at any given level, in the tubal ridge. This is a strip of tall coelomic epithelium overlying the Müllerian duct and forming the caudal continuation of the area of origin of that duct. Here, too, lattice fibers appear within the epithelium, leaving free only a thin layer near the free border (FIGURES 2*f* and 3*b*). Again, a new basal membrane appears between the portions with and without lattice fibers, and the old basal membrane disappears; another portion of what had been epithelium has been transformed into mesenchyme (FIGURES 2*a* and 2*g*).[1, 4]

All these observations are obvious and easily followed in chick embryos (FIGURE 3). Findings in human embryos strongly suggest that, in man, the course of events is essentially the same.[4] An example of a human stage resembling that just described in the chick is shown in FIGURE 4*a*. The Müllerian and Wolffian ducts are separated by mesenchyme, which is enclosed in a basal membrane extending from one duct to the other, much as seen in the chick in FIGURE 3*a*. In an older embryo the Müllerian duct is surrounded by a layer of large fibroblasts with very scant lattice fibers between them (FIGURE 3*b* and 3*c*). Whether the existence of this layer (which is not the primordium of a muscular coat) bears any relationship to the origin of mesenchyme from the duct has not been determined.

Thus a large part of the mesenchyme of the uterovaginal canal develops by transformation of previously well-differentiated epithelia, namely, the Müllerian duct itself and the tubal ridge of the coelomic lining, the future pleura and peritoneum. This mesenchyme forms undetermined portions of the endometrial stroma and myometrium, as well as parts of the corresponding layers of tubes and vagina. Replacement of the Müllerian epithelium by that of the urogenital sinus in the area of the vagina occurs later and has no effect on the origin of the mesenchyme.

Epithelial and mesenchymal tissues of the uterus and tubes are therefore much more closely related to each other in their development, and presumably in their potentialities, than in other organs. Pathology, particularly oncology of the genital tract, including the uterus, is replete with examples of histological differentiation that has mixed or intermediate properties as far as epithelial and nonepithelial stigmata are concerned. Whether or not normal or pathological endometrial epithelium can arise from mesenchymal tissues has not been established.

Considerations based on descriptive embryology frequently have been used in explaining and classifying pathological growths, particularly in the genital tract. In these efforts, alleged differences in embryonic origin of various tissue components have been used as a basis of classification. When one considers what has been shown here, it would seem more appropriate to use embryological knowledge to explain similarities rather than differences. Classification might be undertaken better on the basis of morphological and functional criteria actually observed than on hypothetical differences in origin.[5]

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STEROID HORMONES AND UTERINE GROWTH*

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Numerous studies from this laboratory have been concerned with the role of steroid hormones in the promotion of uterine growth. These studies have emphasized the several interactions that may occur between hormones from the adenohypophysis, the suprarenal cortex, and the ovary which modify the responses of the endocrine receptor organs of the female reproductive tract.[1, 2] Experimental evidence at hand strongly suggests that the metabolic alterations of the female reproductive system are not truly the action of one or two hormones only. It appears more likely that the observed metabolic alterations represent the physiological expression of the sum total of all present biological hormones and/or their metabolites acting in concert on the receptor organs.[3-5]

Previously it was reported that several interactions occur among the estrogens in uterine growth.[6, 7] More recently it was demonstrated that testosterone,[3] cortisone and cortisols,[8-11] and *small* doses of progesterone suppressed to a measurable degree the response of the uterus of the ovariectomized rat to estradiol-17β.

While the aforementioned reports indicated that numerous steroids interfered with the action(s) of estradiol-17β-induced uterine growth in the ovariectomized rat, the question yet remained as to the mechanism(s) involved. Therefore, it seemed of interest to re-evaluate (*1*) the steroids involved in uterine growth, (2) the steroids that interfere with estradiol-17-β-induced uterine growth, and (3) the role of the adenohypophysis and the adrenal glands in uterine growth as well as during the inhibitory process.

More specifically the present report is concerned directly with ascertaining the following: (1) the uterine growth-promoting action of estradiol-17β and its five urinary metabolites (estrone, 16α-hydroxyestrone, 16β-hydroxyestrone, estriol, and 16-*epi*estriol); (2) the influence of progesterone and several of its C-21 related metabolites on uterine growth; (3) the action of testosterone, glucocorticoids, and mineralocorticoids on uterine growth; (4) interactions of ovarian, testicular, and adrenal hormones on uterine growth; and (5) a number of experiments designed to elucidate the mechanism(s) involved in the steroidally induced suppression of estradiol-17β.

EXPERIMENTAL PROCEDURES

Adult albino rats 90 days of age, purchased from the Charles River Breeding Laboratories, Wilmington, Mass., and weighing approximately 200 gm., were used in all experiments involving ovariectomized rats. In experiments utilizing ovariectomized-adrenalectomized and ovariectomized-adrenalectomized-hypophysectomized rats, it was found more suitable to use rats 105 days of age

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and weighing 235 gm. These animals survived the triple surgery much better than the younger rats.

All animals were started on experiment 7 days after ovariectomy. The hypophysectomies were performed 1 week prior to the ovariectomies, and the adrenalectomies were started 3 to 5 min. after the removal of the ovaries.

The ovariectomized rats were housed 6 to a 9 × 9 × 18 inch wire-meshed cage, and were fed the standard Purina Lab Chow and tap water ad libitum. The adrenalectomized-ovariectomized rats were similarly maintained, except for the substitution of 1 per cent NaCl drinking solution in place of the tap water. The ovariectomized-adrenalectomized-hypophysectomized rats received the Purina chow, were maintained in similar cages, as described above, and received 2 drinking solutions: 1 per cent NaCl and 1 per cent glucose.

The hormones were dissolved in sesame oil; each dosage of hormone was

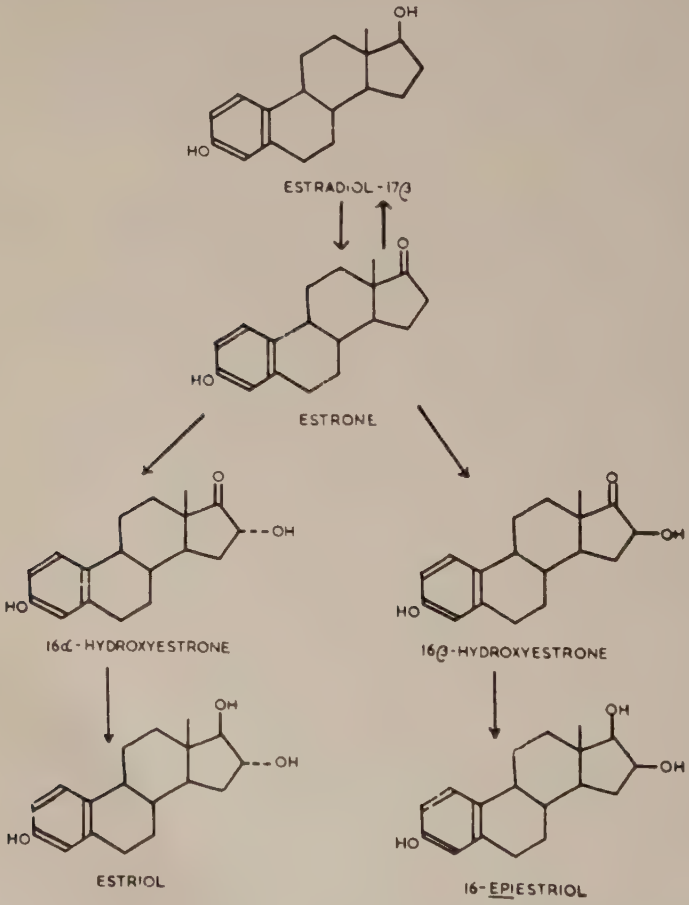

FIGURE 1. Hypothetical scheme for the interrelationship of the estrogens in human urine.

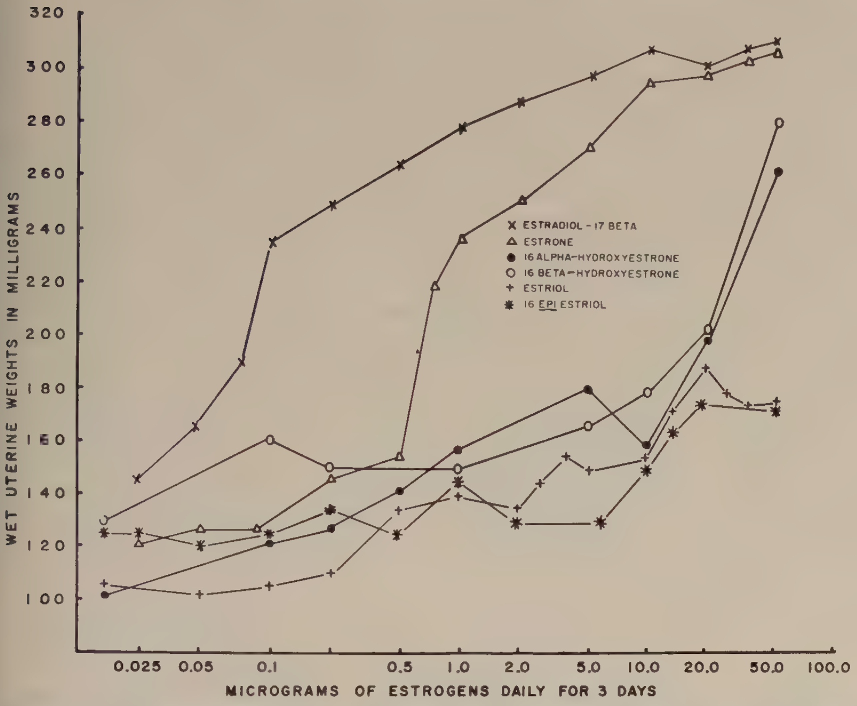

FIGURE 2. Dosage-response curves, showing the comparative effectiveness of estradiol-17β, estrone, 16α- and 16β-hydroxyestrones, estriol, and 16-*epi*estriol. Rats 90 days of age were ovariectomized and, beginning 7 days later, were given 1 subcutaneous injection of estrogen daily for 3 days. Necropsies were performed 24 hours after the last injection. Each point on these and subsequent curves represents the arithmetical mean of uterine weight for at least 11 to 20 animals. The average wet uterine weight for nontreated ovariectomized rats was 118.0 ± 7.0 mg.

contained in either a 0.1 or 0.2 cc. vehicle, and injected subcutaneously daily for 3 days. When 2 or more hormones were injected, they were administered at separate sites. Seventy-two hours after the initial injection(s), necropsies were performed, and the uteri were removed and weighed to the nearest 0.2 mg. on a Roller-Smith torsion balance. Parallel quantitative data were obtained in each experiment on wet and dry weights of the uterus, percentage of water, total nitrogen, and solids within the uterus. Although only uterine weights are used for the purpose of this paper, their presentation is in essential agreement with the biochemical data. The curves depicted for dry uterine weights follow those of the total nitrogen data quite precisely.

RESULTS

Uterine Growth-Promoting Action of the Six Naturally Occurring Estrogens

A great deal of biochemical research has been in progress on the urinary estrogens and their quantitative determination by Marrian and his associates,[13-20] Bauld,[21,22] and Brown.[23,24] FIGURE 1, depicting a hypothetical scheme for the interrelationship of the estrogens in the urine, represents a summary of Marrian's research, which has led us to a clearer concept of the biochemical career of estradiol-17β and the $\Delta^{1,3,5}$ estratrienes. This figure indicates that estrone may undergo hydroxylation at the 16α and 16β position, and that the two hydroxyestrones may then be reduced at C-17 to give estriol and 16-*epi*estriol, respectively. Up to 1954 the three different but closely chemically related estrogens isolated from the urine of nonpregnant and pregnant women were estradiol-17β ⇌ estrone → estriol. These estrogens were characterized biologically by numerous investigators[6] and, up to 1954, these were the only naturally occurring estrogens that were biologically characterized and assayed. The recent availability of 16α- and 16β-hydroxyestrones (thanks to Thomas Gallagher) and 16-*epi*estriol (thanks to Max N. Huffman) gave impetus to the present re-evaluation of the biological activity of estradiol-17β and five of its metabolites, as depicted in FIGURE 1.

The potentiality of each estrogen for the promotion of uterine growth was determined by establishing a dose-response curve (FIGURES 2 and 3). Maximal uterine growth for the 3-day period was obtained with a daily subcutaneous injection of 1.0 μg. estradiol-17β, 5.0 μg. estrone, 50.0 μg. 16α-hydroxyestrone,

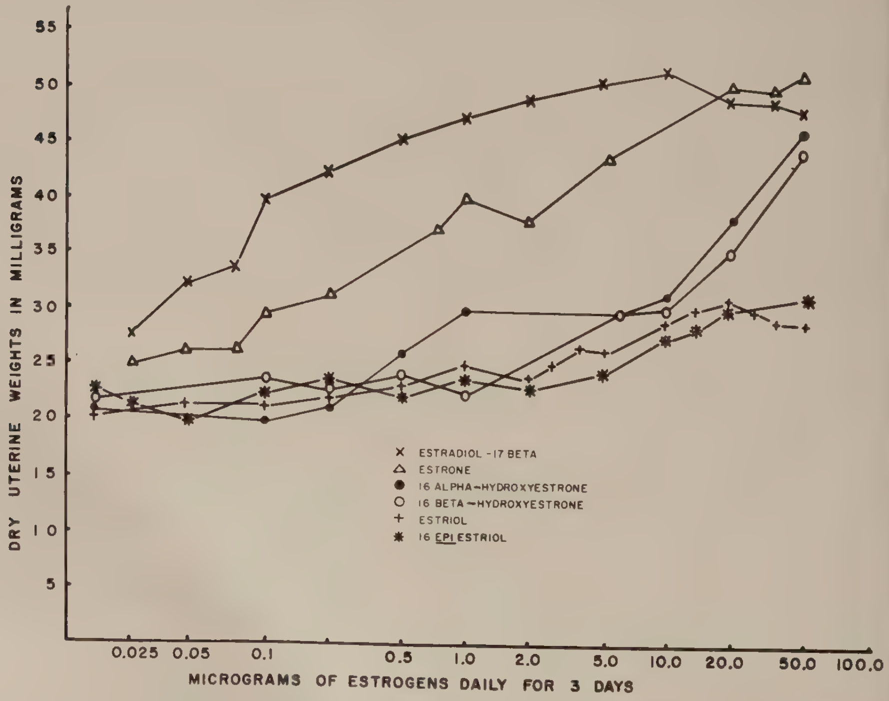

FIGURE 3. Uterine dry weights of the corresponding uteri in FIGURE 2. The dry weights parallel the growth responses indicated by the uterine wet weights and the nitrogen determinations. The dry uterine-weight average for the nontreated ovariectomized rats was 22.0 ± 1.1 mg.

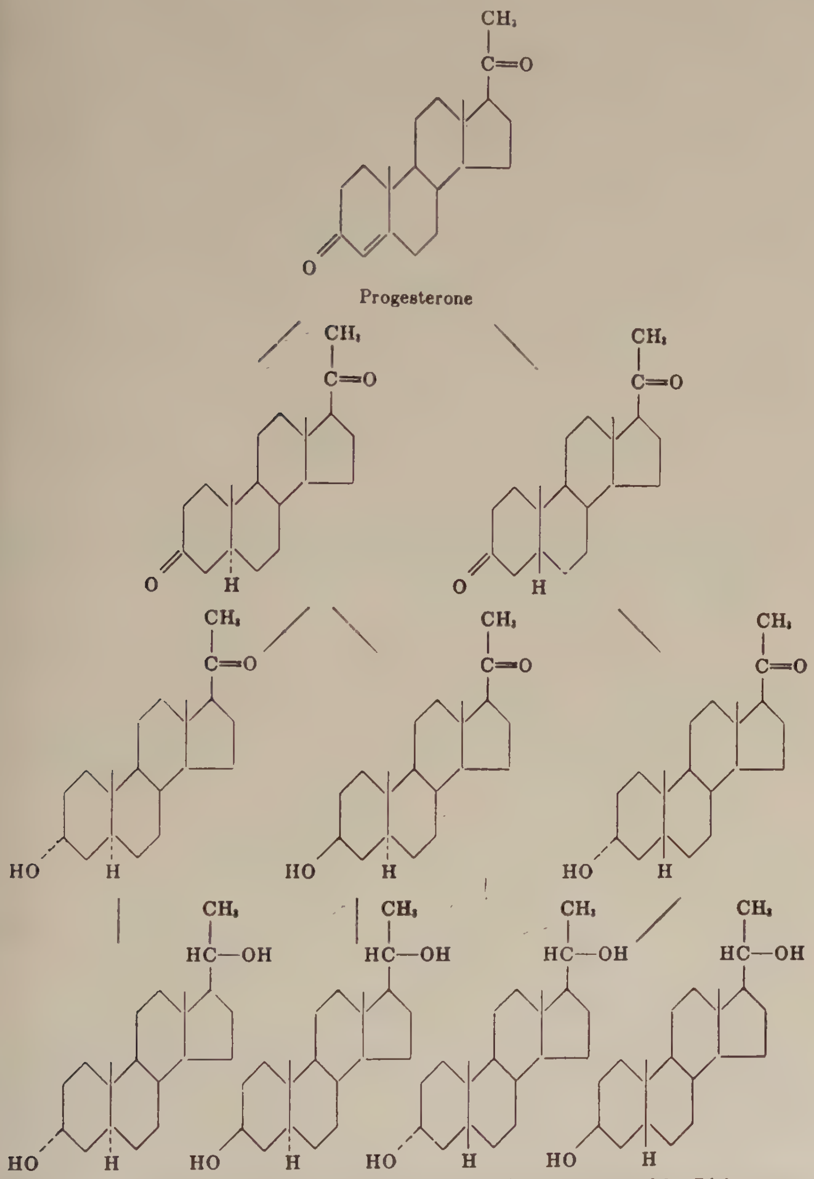

FIGURE 4. Progesterone and its C-21 related metabolites as proposed by Lieberman and Teich.[25]

50.0 μg. 16β-hydroxyestrone, 20.0 μg. estriol, and 20.0 μg. 16-*epi*estriol. Daily doses as high as 50.0 μg. estriol and 16-*epi*estriol proved less effective than the response elicited with 20.0 μg. of the 2 estriols, respectively (FIGURES 2 and 3). Analysis of the biochemical data at hand reveals that the increase in uterine luminal fluid, nitrogen, and solid content of the uteri parallels quite closely the curves depicted for estradiol-17β and its 5 metabolites. These data show that estradiol-17β > estrone > 16α-hydroxyestrone > 16β-hydroxyestrone > estriol > 16-*epi*estriol in the promotion of uterine growth.

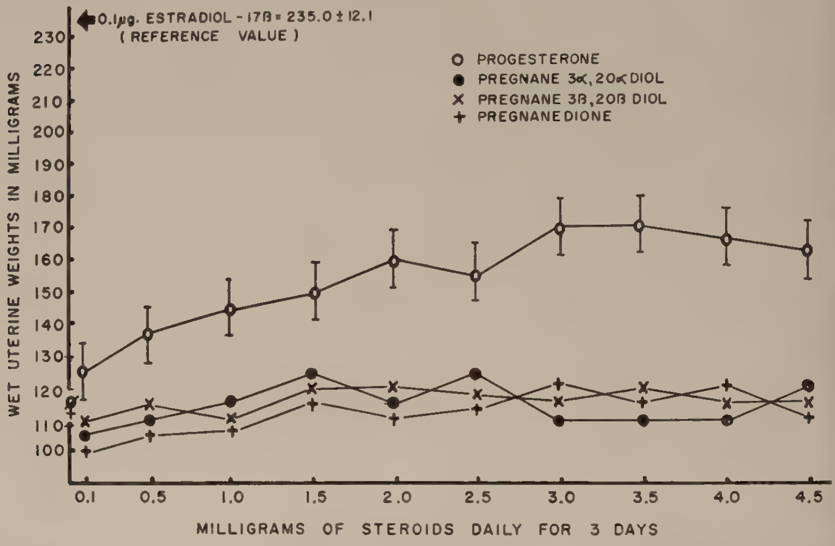

FIGURE 5. Influence of progesterone and pregnanes on uterine growth. The vertical bars on the progesterone curve and subsequent curves represent the standard errors of the arithmetical mean.

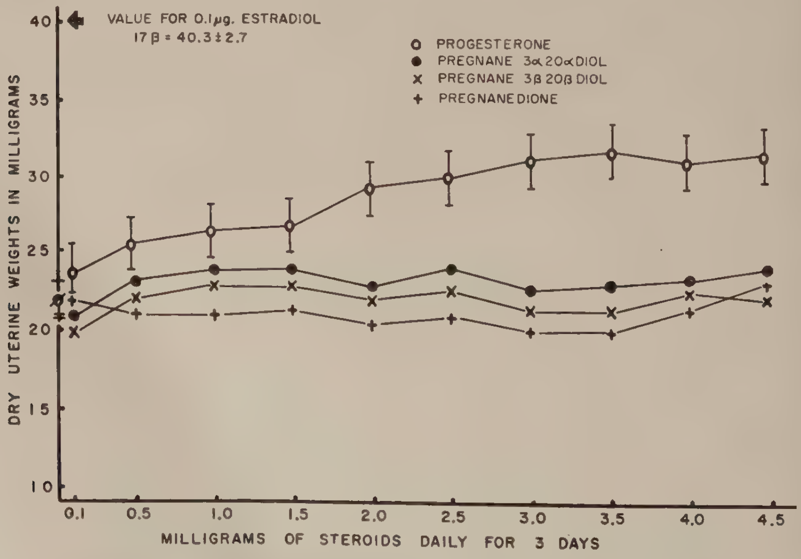

FIGURE 6. Uterine dry weights of the corresponding uteri in FIGURE 5.

ction of Progesterone, Pregnanediols, and Pregnanediones on Uterine Growth

Considerably more biochemical and physiological data are available for the trogens than for progesterone and its C-21 related metabolites. Recently eiberman and Teich[25] described the C-21 metabolites related to progesterone IGURE 4). More recently, Zander[26] has isolated two new progestins from the ood of human females: Δ^4, 3 keto-pregnene 20α ol and Δ^4, 3 keto-pregnene)β ol. Unfortunately the newly reported progestins are not yet available for orough biological assessment. Therefore the present report will be con- rned with the action of progesterone and several of the pregnanediols and egnanediones on uterine growth. Progesterone displays a slight uterine owth-promoting effect. None of the C-21 related metabolites of progesterone us far tested for uterotropic effect induced uterine growth (FIGURES 5 and .

Influence of Testosterone, Glucocorticoids, and Mineralocorticoids upon Uterine Growth

Testosterone in daily doses of 2.0 to 5.0 mg. effected uterine growth in a way distinguishable from that produced by 3 daily subcutaneous injections of 0.1 g. estradiol-17β (FIGURES 2, 3, 7, and 8). Of the 7 glucocorticoids and mineral- corticoids tested [desoxycorticosterone acetate; cortisone (E); hydrocortisone

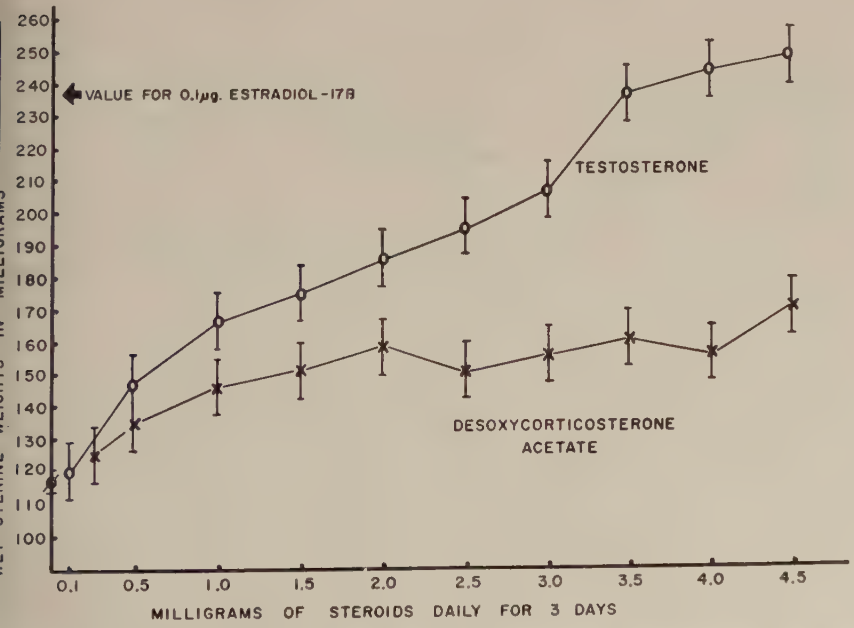

FIGURE 7. Uterine growth-promoting action of testosterone and desoxycorticosterone etate.

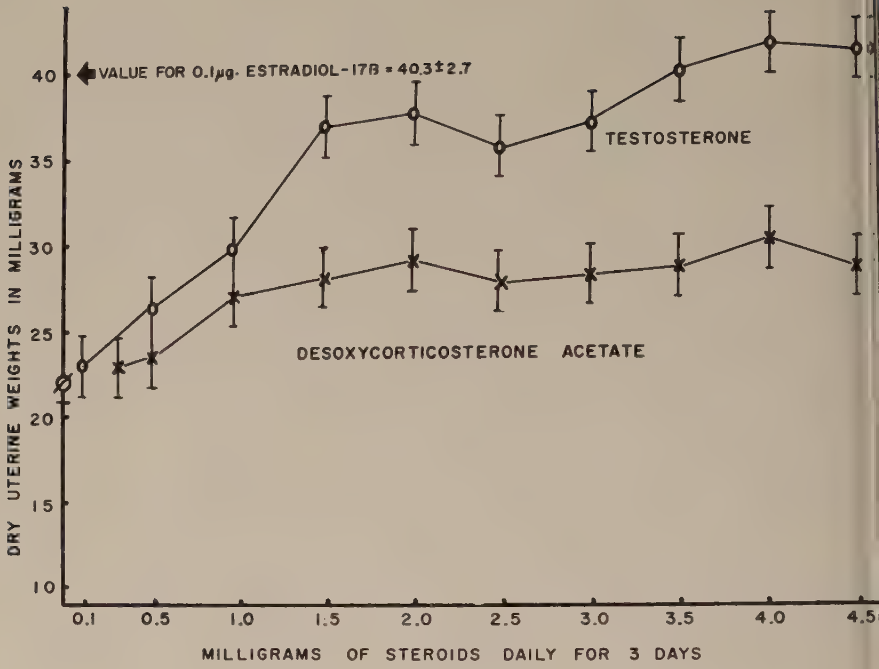

FIGURE 8. Uterine dry weights of the corresponding uteri in FIGURE 7.

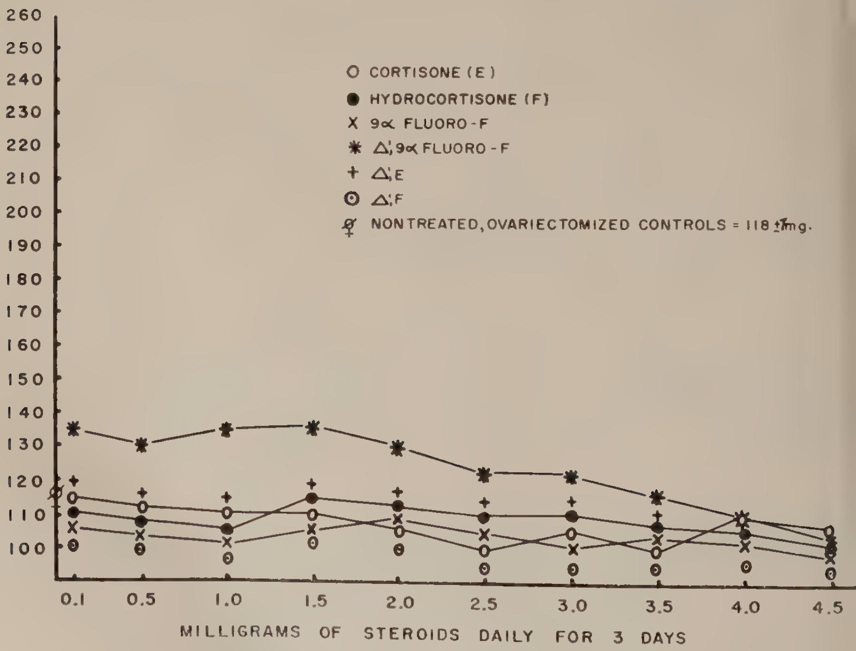

FIGURE 9. Inability of cortisone and cortisols to induce uterine growth.

); Δ^1, F; Δ^1, E; 9αFl F; and Δ^1, 9αFl F], it was observed that desoxycorticos-
rone was the only corticosteroid that displayed the ability to induce *slight*
erine growth (FIGURES 7, 8, and 9).

Interactions of Ovarian, Testicular, and Adrenal Hormones on Uterine Growth

Interactions among the estrogens. Previously, Hisaw *et al.*[6] and Velardo and
urgis[7] reported that estriol and 16-*epi*estriol restricted the uterine growth-
omoting action of estradiol-17β. The recent availability of 16α- and β-hy-
oxyestrones provided the impetus for the current extension of these earlier
periments. Combinations of one of the two hydroxyestrones and estradiol-
β were administered to ovariectomized rats. Preliminary data at hand
dicate that, although the two hydroxyestrones are comparatively weak in
ducing uterine growth, as are the estriols, they do not seem to interfere with
e action of estradiol-17β. Additional studies involving the action of a wide
se range of the hydroxyestrones on the response of 0.1 μg. estradiol-17β are
w in progress.

Interactions of estradiol-17β and progesterone or pregnanes on uterine growth.
ogesterone in low doses ($<$2.5 mg.) interferes with the response of the uterus
the ovariectomized rat to 0.1 μg. estradiol-17β, but larger doses (3.5 to 4.5
g.) do not seem to have an appreciable effect on estradiol (FIGURES 10 and
). None of the pregnanes tested thus far interfered with the uterine growth-
omoting action of estradiol.

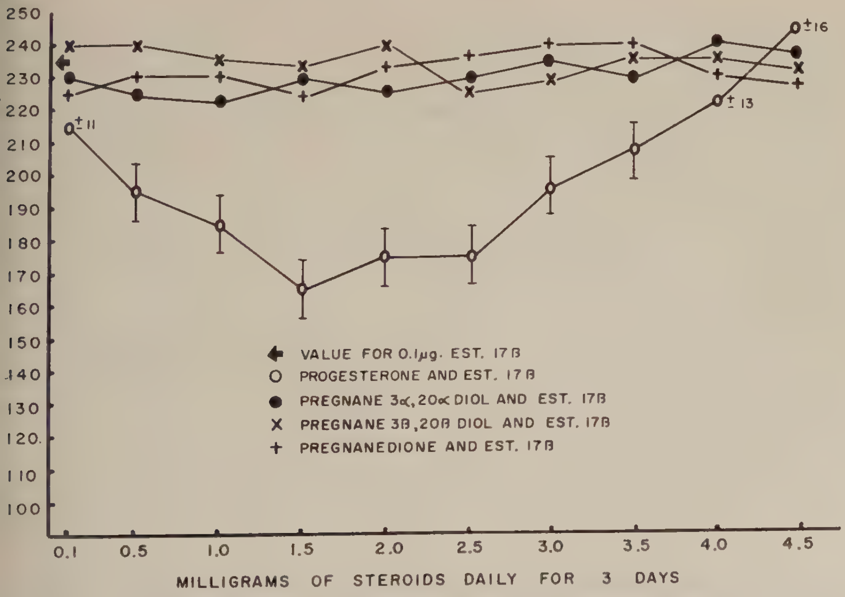

FIGURE 10. Restrictive action of progesterone and the nonrestrictive action of the
egnanes on estradiol in uterine growth.

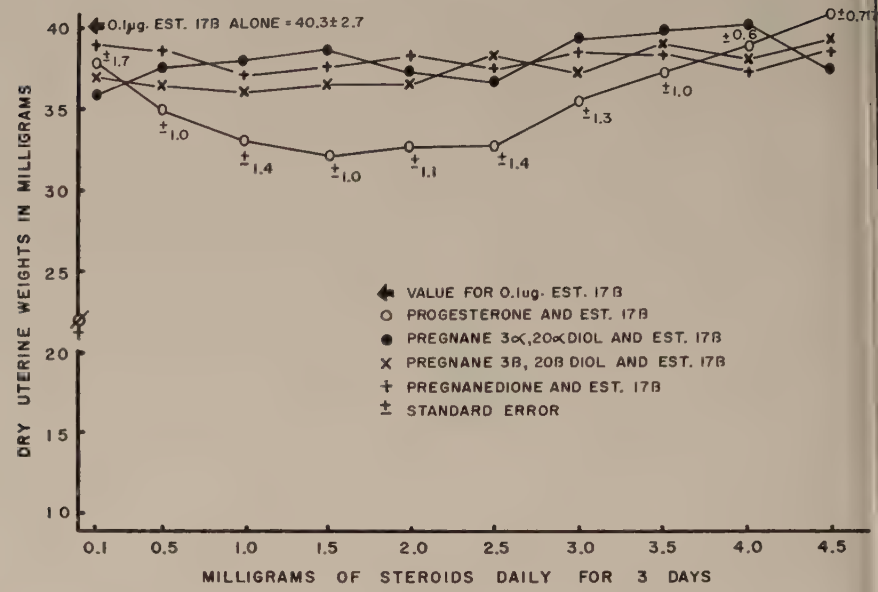

FIGURE 11. Uterine dry weights of the corresponding uteri represented in FIGURE 10.

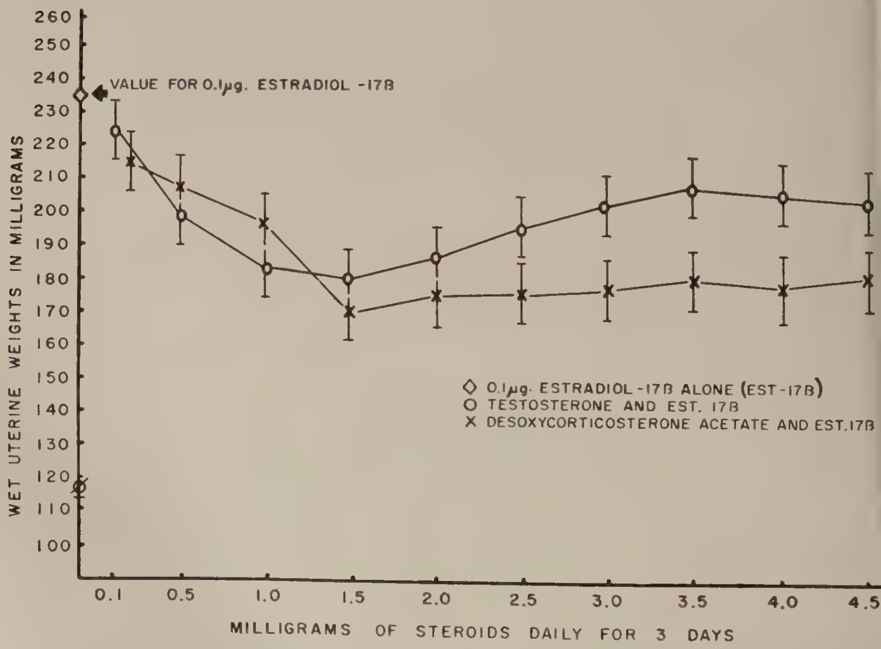

FIGURE 12. Inhibitory action of testosterone and desoxycorticosterone acetate on estradiol in uterine growth.

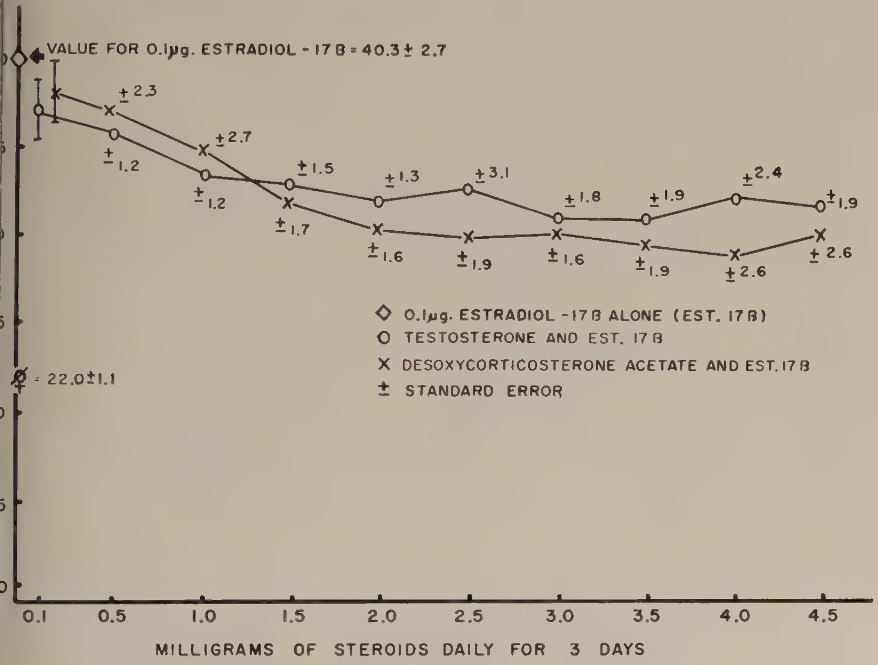

FIGURE 13. Uterine dry weights of the corresponding uteri represented in FIGURE 12.

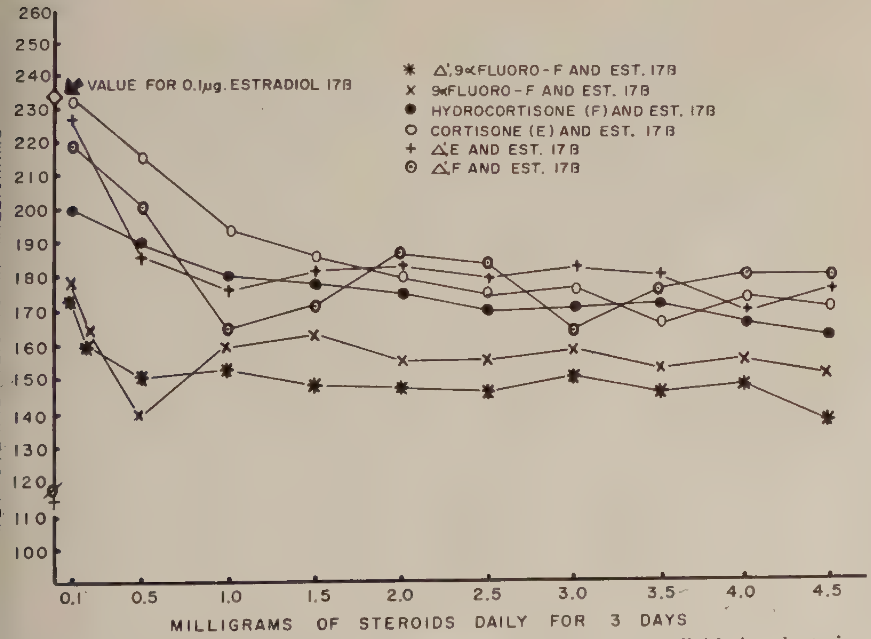

FIGURE 14. Suppressive effects of cortisones and cortisols on estradiol-induced uterine rowth.

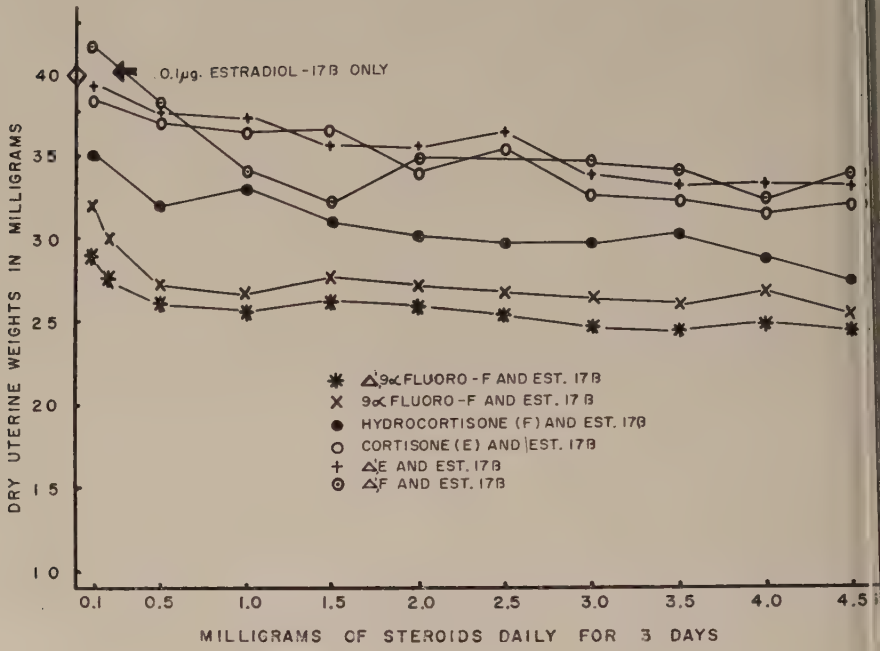

FIGURE 15. Uterine dry weights of the corresponding uteri depicted in FIGURE 14.

Inhibitory action of testosterone and desoxycorticosterone acetate (DCA) on estradiol. These data reveal that both testosterone and DCA display similar inhibitory activity on estradiol-17β. Three daily doses of 0.5 to 1.5 mg. of either testosterone or DCA decreased the uterine response from 235.0 mg. down to 170 to 180 mg., a significant reduction in uterine growth. Doses above 1.5 mg. of either of the two steroids did not induce a greater suppressive effect on estradiol (FIGURES 12 and 13).

Suppressive effects of cortisones and cortisols on estradiol-induced uterine growth. All of the six cortisones and cortisols tested interfered with estradiol. Comparatively, these data reveal that Δ^1, 9αFl F > 9α Fl F > F > E > Δ^1E and Δ^1F in restricting the uterine growth-promoting action of estradiol. It is of further interest to note that both structural modification of the geometry of ring A of compound F and halogenation at the ninth carbon enhance the suppressive effects of cortisol (FIGURES 14 and 15).

Observations Regarding the Mechanism of Action of the Suppressive Effects of the Different Steroids on Estradiol-17β

Several studies showing the competitive interactions of steroids in reproductive processes have provided a considerable impetus to ascertaining the mechanism of action of the suppressive effects of the different steroids on estradiol-17β.[3-12, 27-31] Thus far, several approaches to this problem have been initiated. These clude studies on (1) the structural specificity of steroids in the growth and hibition of the uterus by Szego and Roberts,[4, 5, 31] Huggins and Jensen,[27-30] nd Velardo;[11] (2) the hormone-enzyme relationships by Bever, Velardo, and isaw[32-35] and Villee and Hagerman;[38] and (3) the endocrine organs involved in he suppression of estradiol-17β-induced uterine growth.[39]

The present results are offered in the nature of a preliminary report on the esearch in progress in this laboratory on the mechanism(s) involved in the estriction of uterine growth. Previously, it was postulated that estriol might timulate the release of adrenocorticotropic hormone (ACTH) and consequently drenocortical steroids. This line of reasoning seemed to support the thought hat the antiuterotropic activity might well be due to the inhibitory effect of the adrenal corticosteroids, an effect known to antagonize estradiol and strone on uterine growth. Thus it seemed that a better insight into the fac-ors operative in uterine growth, as well as in the suppressive effects of the different steroids, might be gained if this assumption were tested.

Results from the present experiments reveal that: (1) estradiol-17β is an active uterine growth-promoting agent in (a) ovariectomized, (b) ovariectomized-adrenalectomized, and (c) ovariectomized-adrenalectomized-hypophysectomized rats; (2) estriol and 16-*epi*estriol both inhibit estradiol-17β-induced uterine growth in (a) ovariectomized rats—from 0.1 μg. estradiol-17β controls of 243 mg. down to 156 mg., (b) ovariectomized-adrenalectomized rats—from

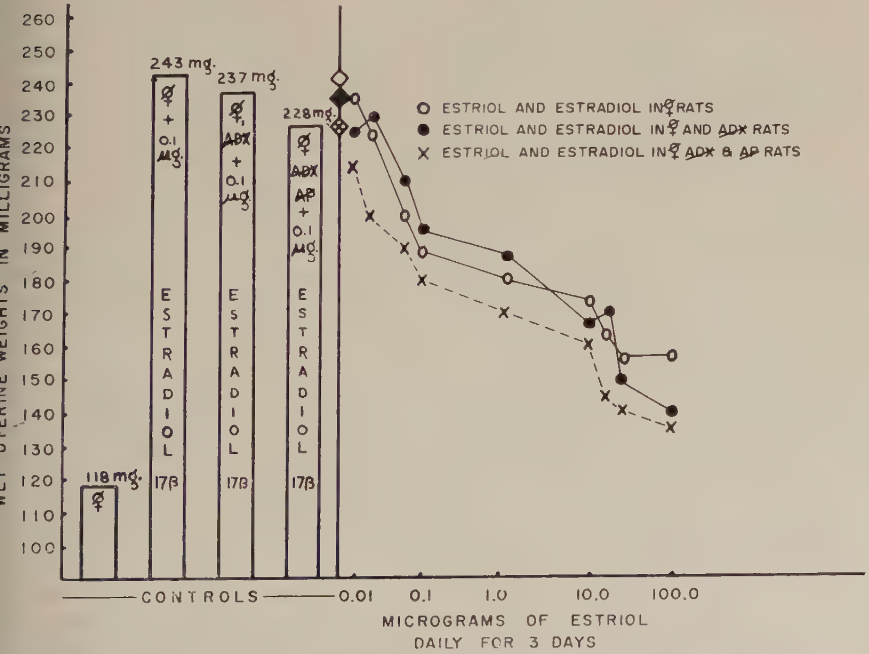

FIGURE 16. Restrictive action of estriol on estradiol-17β-induced uterine growth independent of the adrenal gland and adenohypophysis.

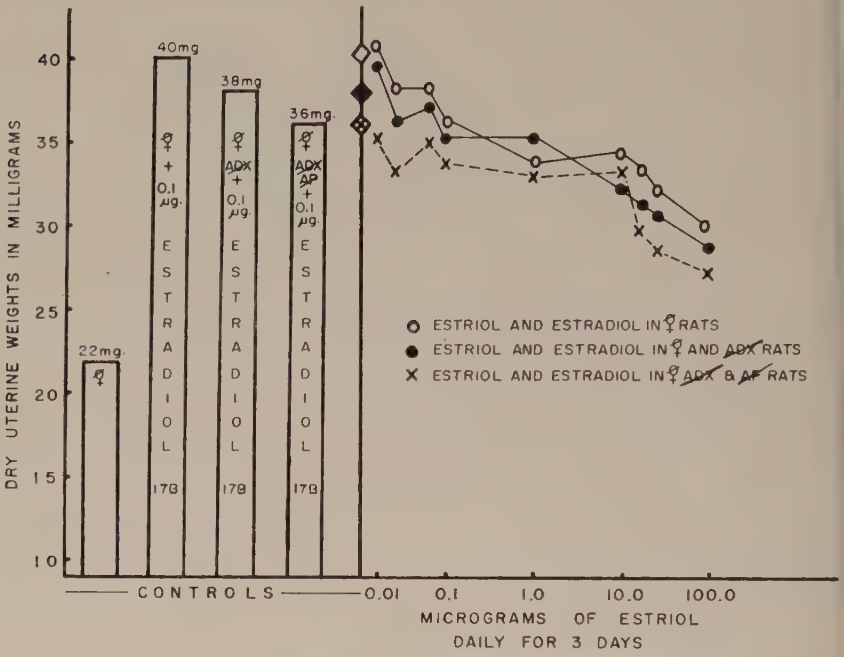

FIGURE 17. Dry-weight data on corresponding uteri depicted in FIGURE 16.

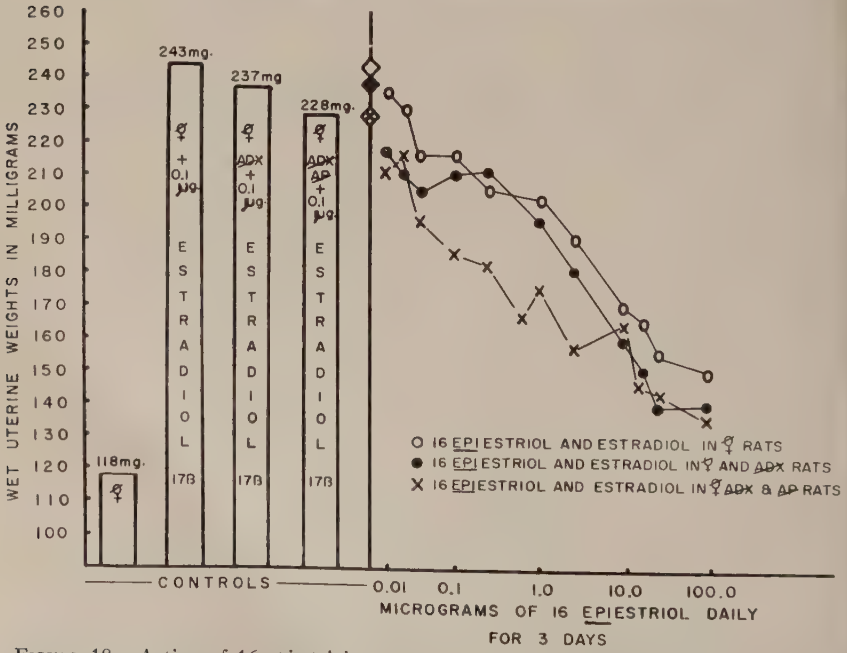

FIGURE 18. Action of 16-*epi*estriol on estradiol-17β in uterine growth not mediated by way of the adrenal glands or the adenohypophysis.

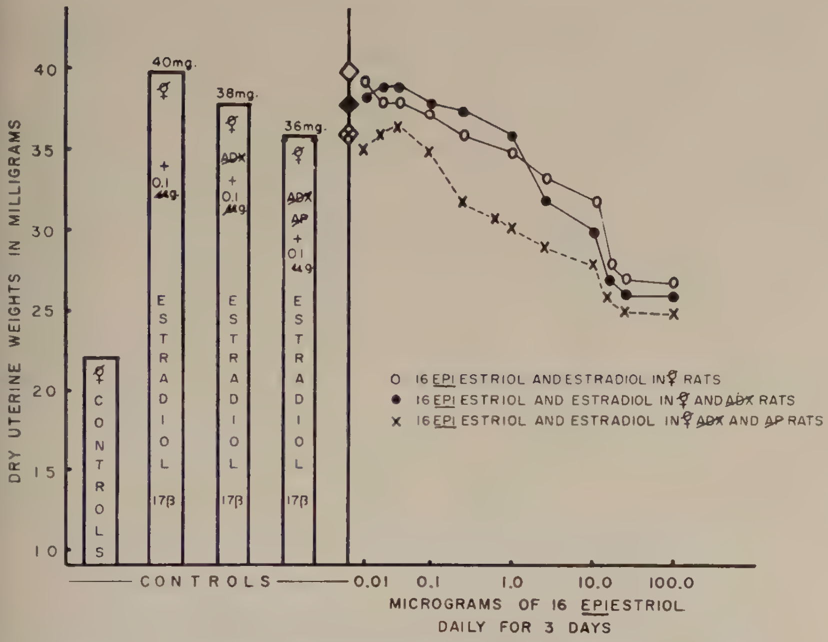

FIGURE 19. Dry-weight data on corresponding uteri represented in FIGURE 18.

237 mg. down to 139 mg., and (c) ovariectomized-adrenalectomized-hypophysectomized rats—from 228 mg. down to 135 mg.; and (3) the suppressive effects of the estriols need not be mediated by way of the adenohypophysis and the adrenal glands (FIGURES 16 to 19).

DISCUSSION

The results of these experiments reveal the nature of the steroids importantly concerned with the growth phase of the uterus, as well as the steroids antagonistic to the uterine growth-promoting action of estradiol-17β. The present report also gives a rather comprehensive view of the numerous interactions that occur among hormones of the ovaries and the suprarenal cortex. Superimposed upon these basic studies is a demonstration that indicates that the suppressive effects of estriol and its isomeric form, 16-*epi*estriol, on estradiol-17β-induced uterine growth are not mediated by way of the adenohypophysis or the suprarenal cortex, but more likely act directly on the endocrine receptor organs.

Courrier[40] and, more recently, Velardo[41, 42] have summarized the numerous actions and interactions of the luteoids and folliculoids. It is well known that progesterone can restrict in part the estrogens in rodents, yet in other species these two ovarian hormones may synergize, for example, the progestational phase of the primate endometrium. On a species-wide basis, much less is known of the actions and interactions of the corticoids, folliculoids, luteiods, and testoids. Information from recent basic studies and clinical observations on the metabolic career of isotopically labeled hormones, coupled with data on the hormonal status of patients with endocrinopathies, offer important leads to our understanding of the hormonal interrelationships in the human female.

It is well known that the therapeutic problem of dealing with several endocrinopathies is complicated by the recognition that a target organ probably does not respond simply to one hormone alone, but more likely to the total internal hormonal environment of the patient. In both frank myxedema and hyperthyroidism there is observed consistently a marked dysfunction of the female reproductive system, manifested either by amenorrhea or menorrhagia. In 1956 Velardo and Sturgis,[8] referring to the work of Goldsmith *et al.*,[43] stated that it is not entirely clear whether thyroid disease causes this abnormality by interfering with the release of gonadotropins from the adenohypophysis, by altering ovarian function, or by a direct effect on the uterine mucosa. Since 1956, Engle *et al.*[44] and Gallagher *et al.*[45] have produced a great deal of helpful information which reveals that a thyroid hormone, triiodothyronine, has a marked effect upon the metabolism of the precursor of estradiol-17β, namely, testosterone. Gallagher and his associates have shown that triiodothyronine regulates the conversion of testosterone to androsterone and etiocholanolone. It is of direct interest to point out that androsterone is a biologically active androgen. These facts suggest a more complex interrelationship than hitherto evisaged. Recent evidence indicates that the gonadal hormones may well be influenced by way of the hypothalamico-pituitary-thyroidal-adrenal-gonadal axis. This thought is consonant with the thesis that the several metabolic alterations of the female reproductive tract are governed by the sum total of the biologically active hormones *and/or* their metabolites acting *in concert.*

Another clinical problem suggesting the interactions of hormones is the Stein-Leventhal syndrome of polycystic ovaries. During the past few years a considerable amount of evidence has been accumulating to show that the Stein-Leventhal syndrome is another clinical endocrinopathy that may in similar fashion involve more than one system of glands of internal secretion. The direct surgical approach on the ovaries, as recommended by Stein and Cohen,[46] suggests that this disease is limited to the ovaries, but ovarian wedge resection did not invariably reverse the clinical career of the disease. Several subsequent reports have indicated the possibility that in some cases adrenal hyperfunction may be basically involved in the several clinical entities associated with the Stein-Leventhal syndrome: hirsutism, obesity, ovarian lesions, and endometrial activity.[47, 48] In this complicated picture it is extremely difficult for the clinican to attack with a high degree of assurance only one of the several possible sites of the endocrine disorder that may contribute to the clinical complaints of obesity, hirsutism, amenorrhea, and sterility. It therefor seems logical to approach this problem through a series of hormonal tests, the results of which may be useful as a basis for a rational combination of therapeutic regimens.

Furthermore, the demonstration that cortisone therapy reduces the urinary estrogens in patients with mammary cancer adds further credence to the thought that there are numerous interactions among hormones of the adrenal cortex and the ovary.[49] Kupperman and his associates[50] have reported recently that, in addition to mechanical barriers, one must consider that thyroid, adrenocortical, and ovarian disturbances also may cause infertility in women. These observations suggest that the several induced metabolic alterations of the female reproductive tract in lower mammals may represent a similarity of expression consistent with and diagnostic of the clinically recognizable symptomatology of reproductive dysfunction.

It is rather difficult to define with clarity the complex hormonal interactions that contribute to the formation of certain recognizable clinical syndromes. Biochemical determinations of hormones and their metabolites offer a chance to separate several of these complicated patterns into their component parts, and thereby call attention to functional and dysfunctional pathways that otherwise may be overlooked. In this connection it should be mentioned that the splendid contributions by Gallagher and Engel serve as long awaited breakthroughs in the clarification of certain of the metabolic pathways and precursors of the sex hormones in the human female.

In the final analysis, these data suggest that (1) uterine growth and the attendant metabolic alterations associated therewith are governed by the circulating hormones and their metabolites in a physiological system at a specific time; (2) the estrogens exert their effects *in concert*; and (3) numerous interactions occur among (a) the estrogens, (b) the estrogens and progesterone (c) the estrogens and testosterone, and (d) the estrogens and adrenocorticosteroids. Thus, it seems that the hormonal action observed is not the result of one hormone alone; more specifically, it appears that the metabolic alterations of the uterus are due to *all* of the hormones and their metabolites acting *in concert.*

SUMMARY

(1) A detailed evaluation of the steroid hormones involved in (a) uterine growth and (b) suppression of the uterine growth-promoting action of estradiol-17β was undertaken in an effort to clarify the endocrines, hormones, and factors operative in the growth and inhibition of uterine proliferation. The role of the endocrine organs involved in the restrictive actions of estriol and 16-*epi*estriol was also carefully studied in this respect. It accordingly seemed germane to start with an assessment of the uterine growth-promoting action of estradiol-17β and its five urinary metabolites, the luteiods, testoids, and a number of glucocorticoids and mineralocorticoids, as well as the possible interactions of these compounds with estradiol-17β in uterine growth.

(2) The potentiality of each of the naturally occurring estrogens was determined by establishing a dose-response curve. These data reveal that estradiol-17β > estrone > 16α-hydroxyestrone > 16β-hydroxyestrone > estriol > 16-*epi*estriol in the promotion of uterine growth.

(3) Progesterone displays a slight uterine growth-promoting effect in ovariectomized rats. None of the C-21 related metabolites of progesterone [the pregnanediols (3α, 20α; 3α 20β; 3β, 20β), or the pregnanediones (pregnanedione and allopregnanedione)] induced uterine growth.

(4) Testosterone in daily doses of 2.0 to 5.0 mg. for 3 days influences marked uterine growth; daily doses of 3.5 to 5.0 mg. effected uterine weights that were indistinguishable from those produced with 0.10μg. estradiol-17β.

(5) Of the 7 glucocorticoids and mineralocorticoids tested [desoxycorticosterone; 11 dehydro-17 hydroxycorticosterone acetate (E-ACl); 17-hydroxycorticosterone-21 acetate (F-AC); Δ^1F; Δ^1E; 9α Fl F; and $\Delta^1$9α Fl F], it was observed that desoxycorticosterone was the only corticosteroid that displayed the ability to induce (slight) uterine growth.

(6) Numerous interactions exist among the estrogens; both estriol and 16-*epi*estriol display modulating effects upon the uterine growth-promoting action of estradiol-17β and estrone. The possible interactions among the 16α- and β-hydroxyestrones currently are under investigation.

Progesterone at low doses interferes with estradiol-17β-induced uterine growth, but synergizes with estradiol when given in larger doses ($>$2.5 mg. daily).

Testosterone, which displays androgenic, estrogenic, and progestational activity, exerts a marked suppressive effect upon estradiol.

All of the corticoids tested thus far inhibit to a great degree the action of estradiol-17β. Comparatively, these data reveal that $\Delta^1 9\alpha$Fl F $>$ 9α Fl F $>$ F $>$ E $>$ Δ^1E and Δ^1F $>$ DCA in restricting the uterine growth-promoting action of estradiol.

(7) Neither the pituitary nor the adrenal glands are required for mediation of the inhibiting action of the estriols, progesterone, testosterone, and the adrenal cortical steroids on estradiol-17β. Data previously obtained by Bever, Velardo, and Hisaw indicate that the inhibitory effects noted above are associated with differences in effect on the enzyme content of the uterus. These investigators showed that estradiol increases the activity of the lactic dehydrogenase-DPNH oxidase system of the uterus by increasing DPNH, while the other hormones tend to increase the LDH component. Rosa and Velardo have localized histochemically the DPN and SDH reactive areas of the uterus, thus adding credence to the biochemical determinations.

(8) These data suggest that (1) uterine growth and the attendant metabolic alterations associated therewith are governed by the circulating hormones and their metabolites in a physiological system at a specific time; (2) the estrogens exert their effects in concert; and (3) numerous interactions occur among (a) the estrogens, (b) the estrogens and progesterone, (c) the estrogens and testosterone, and (d) the estrogens and adrenocorticosteroids. Thus it seems that the hormonal action observed is not the result of one hormone alone; more specifically it appears that the metabolic alterations of the uterus are due to all of hormones and their metabolites acting in concert.

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Discussion

Leonard J. Lerner* (*Scientific Division, The Wm. S. Merrell Co., Cincinnati, Ohio*): Velardo, referring to the work of Hisaw *et al.*,[1] and of Velardo and Sturgis,[2] has shown that weak estrogens such as estriol or 16-*epi*estriol can partially antagonize the uterotrophic effect of strong estrogens such as estradiol or estrone.

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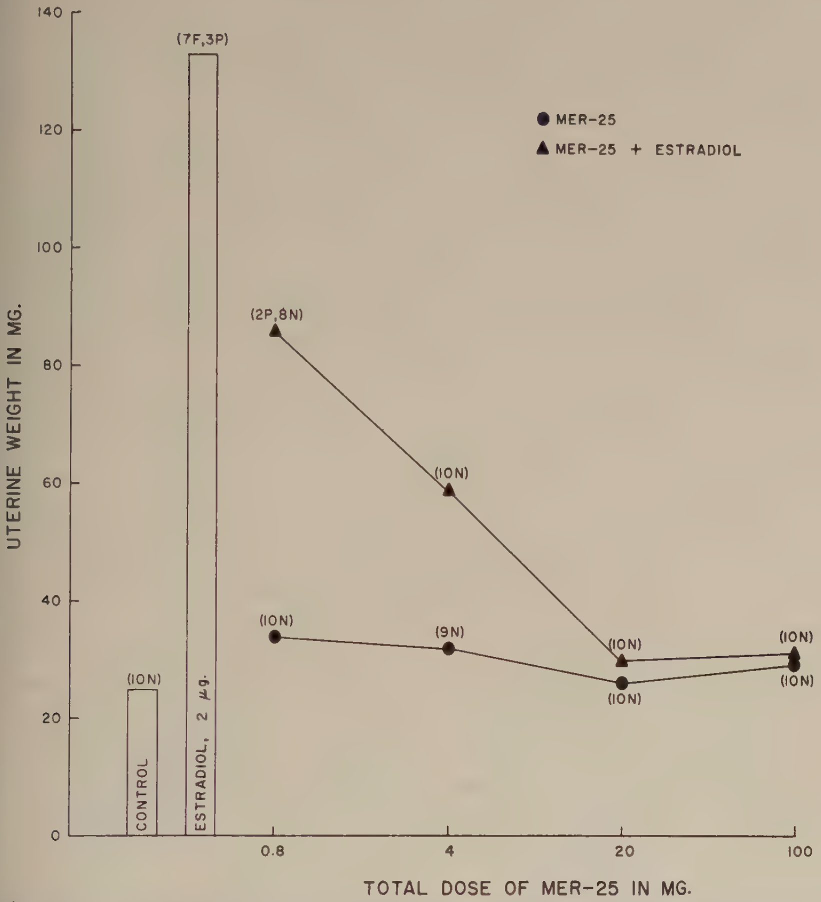

FIGURE 1. Antagonism by MER-25 of the uterine effects of estradiol benzoate in im ature rats. The numbers preceding the letters at each dose point indicate the numbers f animals per luminal fluid grade. The letters indicate the grade: F = full; P = partially lled; N = no fluid.

A nonsteroidal compound, 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-ethoxyphenyl ethanol (MER-25), that completely prevents the uterotrophic esponse to estrogens, is the subject for this brief report. A full report will ppear elsewhere.[3]

Immature female rats weighing approximately 40 gm. were employed. hese animals were injected subcutaneously once daily for 4 days with MER-25 total doses of 0.8, 4, 20, and 100 mg. and in combination with estradiol enzoate in total doses of 2μg. Control animals were administered the olive il vehicle or estradiol benzoate. On the day following the last injection, the nimals were sacrificed. The amount of uterine luminal fluid was estimated, nd the uteri were dissected and the fluid removed before the organ was eighed.

The results of this study are illustrated in Figure 1. MER-25 did not alter uterine weight or luminal fluid. A dose of 20 and 100 mg. of MER-25 completely blocked the uterine effects of 2 μg. of estradiol benzoate and doses of 0.8 and 4 mg. of this compound partially prevented the effects of the estrogen on the uterus.

The availability of this compound not only has obvious clinical implications but may well serve as a tool in the study of uterine and reproductive physiology.

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Part II. Biochemistry and Histochemistry of the Uterus

SOME BIOCHEMICAL ASPECTS OF THE UTERUS*

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Biochemical and histochemical studies of uterine composition following administration of steroids or during the cycles have contributed considerably to our knowlege of hormone action. Estrogen induces an increase in uterine weight in virtually all species; this is associated with hyperemia and is followed by an increase in amino acid incorporation, nucleic acid synthesis, and nitrogen deposition.[1] Estrogen also stimulates phosphorus incorporation, oxidative metabolism, aerobic and anaerobic glycolysis, and glycogen deposition.[2] The importance of glycogen as a source of energy has been suggested. It is well known that glycogen is present in the endometrium of primates[3] and that estrogen will increase the glycogen in the endometrium and myometrium. This carbohydrate, however, is largely in the muscle of rats and mice.[4] Enzymes such as β glucuronidase[5] and alkaline phosphatase,[6] to mention only two, respond to estrogen with an increase in concentration. Progesterone administration to the previously primed animal induces morphologic changes. When a secretory endometrium is developed, an increase in glycogen and nucleic acids is observed. Lutwak-Mann[7] has recently correlated the progestational endometrial morphology with an increase in carbonic anhydrase.

Despite the interest in uterine steroid responsiveness, on occasion one must seek with diligence for data concerning one species, not to mention the effect of age and altered physiological states upon hormone action. It is not within the scope of this paper to relate biochemical and histochemical data from many species, but rather to confine our considerations primarily to the mouse and rat. This position is partially justified, possibly by the knowledge (1) that carbohydrate use by the guinea pig and rat uterus differ,[2] and (2) that uterine glycogen concentration is doubled by estrogen in mice and rats, but is unchanged in hamsters and guinea pigs.[8] The response to estrogen among the rodents is not uniform. Current studies were designed to explore (1) the influence of steroids on the composition of the immature mouse uterus, (2) cyclic changes in uterine composition of adult mice, and (3) the effect of thiouracil and chorionic gonadotropin on the rat uterus.

Methods

The following procedures of analysis were used. The uteri were dissected free of mesentery and fat, and any contained water was blotted on filter paper. For estimation of water content, the uteri were dried to constant weight at

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Acknowledgment is made to CIBA Pharmaceutical Products Inc., Summit, N. J., for testosterone propionate (Perandren), estradiol dipropionate (Ovocylin dipropionate), and progesterone (Lutocylin), and to E. R. Squibb & Sons for chorionic gonadotropin (Follutein).

95° C. The dry tissue was used for estimation of lipid and nitrogen by methods previously presented.[9] Nitrogen values were converted to protein by use of the factor 6.25. Fresh uterine tissue was used to estimate alkaline phosphatase,[10] β-glucuronidase,[11] ascorbic acid,[12] glycogen,[13] lactic acid,[14] and cholesterol.[15]

Uterine Response in Immature Mice

Estrogen is known to increase mouse uterine water, nucleic acids,[16] glycogen,[8] and β-glucuronidase,[5] and to enhance the incorporation of acetate into cholesterol.[17] Estrogen increases alkaline phosphatase[6] and depletes epithelial lipid in the uterus, as estimated histochemically.[18] Ascorbic acid reappears in the uterus of the castrated rat following estrogen.[19] Although testosterone propionate increases uterine weight, this steroid may or may not increase β-glucuronidase[20] and does not increase glycogen[21] or alkaline phosphatase histochemically.[22] Progesterone administered to the immature rodent does not increase uterine glycogen,[21] alkaline phosphatase,[22] or β-glucuronidase.[20]

Swiss mice, 21 days old, were given a single subcutaneous injection of either 10 μg. estradiol dipropionate, 1.0 mg. testosterone propionate, or 1.0 mg. progesterone. The mice were autopsied 72 hours later. Estrogen administration increased uterine water from 78.1 per cent to 84.8 per cent and also increased the dry weight of the organ so that the wet:dry weight ratio increased from 4.5:1 to 6.6:1. Testosterone propionate had a lesser effect on uterine weight, and water content increased to only 80.6 per cent. Nevertheless, the wet:dry weight ratio of the uterus increased to 5.2:1. Progesterone did not enhance uterine weight in the dosage used, but did reduce uterine water content (TABLE 1).

A significant increase in total protein and protein concentration of the dry uterine tissue followed estrogen and testosterone administration. Lipid concentration decreased, but total lipid increased due to the increase in uterine mass following estrogen stimulation. The decrease in lipid concentration imposed by testosterone propionate did not alter total uterine lipid. Progesterone increased protein concentration without increasing total protein of the dry uterine tissue, and did not influence lipid. Protein of the dry uterine tissue was increased by estradiol dipropionate from 2.15 mg. in controls to 6.62 mg., and by testosterone propionate to 3.42, but was decreased slightly by progesterone to 1.89 mg. Despite uterine tissue growth induced by estrogen, the proportions of protein and lipid in the fresh wet tissue were less than those in control tissues.

Alkaline phosphatase enzyme concentration was increased sixfold by estradiol dipropionate, but androgen and progesterone were without effect. Since uterine weight was stimulated by testosterone propionate, total alkaline phosphatase was increased (TABLE 1).

Glucuronidase enzyme concentration in the mouse uterus was increased significantly by both the estrogen and the androgen. The increased β-glucuronidase enzyme concentration following progesterone was of borderline significance (TABLE 1).

TABLE 1

INFLUENCE OF STEROIDS ON IMMATURE MOUSE UTERI

	Control	Estradiol dipropionate	Testosterone propionate	Progesterone
No. mice	128	57	52	74
Uterine wt. (mg.)	14.5	58.4	24.1	10.4
Dry wt.	3.1	8.9	4.6	2.6
Water (%)	78.1	84.8	80.6	74.8
Protein (%)	69.2	74.4	74.3	72.3
Lipid (%)	29.6	27.7	26.9	30.1
Alkaline phosphatase				
No. mice	13	10	9	9
Uterine wt. (mg.)	8.6	30.6	22.4	12.4
μg./mg./hr.	1.52 ± 0.17	9.7 ± 0.2	1.38 ± 0.17	1.41 ± 0.18
Total	14.2 ± 1.9	296.9 ± 28.2	31.2 ± 4.3	15.8 ± 1.2
β-Glucuronidase				
No. mice	15	9	10	12
Uterine wt. (mg.)	11.7	47.7	20.4	11.2
Units/gm./hr.	653 ± 60	1779 ± 175	1560 ± 108	9680 ± 81
Total	7.3	57.7	36.2	11.6
Ascorbic acid				
No. mice	12	8	10	9
Uterine wt. (mg.)	9.4	28.4	30.3	12.1
μg./mg.	0.54 ± 0.17	0.12 ± 0.05	0.23 ± 0.07	0.51 ± 0.18
Total	5.0	3.8	7.0	5.9
Cholesterol				
No. mice	32	14	8	9
Uterine wt. (mg.)	13.9	60.0	22.1	15.1
Per cent free	0.26 ± 0.04	0.19 ± 0.05	0.27 ± 0.06	0.29 ± 0.05
Glycogen				
No. mice	12	12	12	
Uterine wt. (mg.)	14.2	38.1	24.9	
Per cent	0.27 ± 0.04	0.41 ± 0.06	0.38 ± 0.03	

Estradiol dipropionate and testosterone propionate decreased uterine ascorbic acid and increased uterine glycogen concentration in the mouse. Progesterone did not influence ascorbic acid levels in the mouse uterus. Cholesterol concentration was not significantly increased by hormonal treatment. The cholesterol was essentially all free cholesterol and was increased in total amount with the hormonally induced increases in uterine weight (TABLE 1).

Many of the influences of estradiol dipropionate on the mouse essentially substantiate previously reported estrogen effects on the uterus of this species or other rodents. The increase in water and wet:dry weight ratio has been noted.[23, 24] An increase in uterine weight is accompanied by an increase in uterine nitrogen in the rat,[23] and an associated increase in nucleic acids in mice[16] and rats.[25] Despite the increase in uterine mass and the synthesis of protein, it was noted that, when calculated on a wet-weight basis, the proportion of mouse uterine protein was reduced from 15.2 to 11.2 per cent by estrogen administration. Estrogen reduced uterine lipid concentration, but increased total lipids in the mouse uterus; phospholipids increase in rat uteri.[26] Our data failed to reveal a change in mouse cholesterol concentration under estrogen stimulation, but total cholesterol increased with the increased mass of tissue. Emmelot and Bosch[17] found a thirtyfold increase in acetate incorporation into cholesterol in the mouse uterus after estradiol.

The increase in β-glucuronidase concentration in the mouse uterus in response to estrogen stimulation is in agreement with earlier studies,[20, 27] but a relationship between this enzyme and the growth process is not clear.[28]

Estrogen stimulation favors the deposition of uterine glycogen. The glycogen is deposited essentially in the myometrium as energy for contraction,[25] and may have resulted from a stimulation of hexokinase.[21] Whether or not the increase in alkaline phosphatase plays a role in the uterine carbohydrate process is not evident.

Ascorbic acid increase following estrogen administration to castrated rats has been reported.[19] However, in the intact mouse, estrogen and androgen decreased ascorbic acid concentration. The role that ascorbic acid could play in oxidation and reduction suggests the need for a further evaluation of its role in the uterus.

Testosterone propionate increases the wet and dry weights of the rat uterus[29] and enhances the uptake of glycine.[30] In the mouse, testosterone propionate increased the wet and dry uterine weights and the protein concentration while reducing lipid concentration. The proportions of protein and lipid in the uterus on a wet-weight basis were only slightly below control levels, in contrast to the effect of estrogen. The increase in uterine weight following androgen was accompanied by an increase in β-glucuronidase, as has been observed in the rat,[20] but alkaline phosphatase concentration was not changed in agreement with histochemical studies.[22] Despite a failure to observe an increase in uterine glycogen in the rat treated for 3 days with 0.5 mg. testosterone propionate,[21] a significant increase in mouse uterine glycogen was induced with the androgen.

Progesterone may induce a uterine water loss[31] even in the immature mouse uterus, and may increase the protein concentration. These alterations in uterine composition following progesterone resulted in an increase of protein from 15.2 to 20.0 per cent on a wet-weight basis. An increase in uterine ribonucleic acid (RNA) due to progesterone has been suggested.[32] Progesterone did not influence alkaline phosphatase concentrations or ascorbic acid in substantiation of previous reports for mice[22] and rats,[17] respectively.

Estrous Cycle in Mice

During the estrous cycle in mice, water content varies,[33] as do oxygen consumption and rate of glycolysis.[34] Anaerobic glycolysis is maximal at proestrus, a time when aerobic glycolysis is minimal. Dirschel *et al.*[35] observed a slight maximum for uterine dry weight at proestrus and estrus, a time when glycogen and RNA also were present in greater amount.[21, 32] The marked effect of estrogen administration on β-glucuronidase in the immature mouse uterus prompted a study of the glucuronidase during the estrous cycle. Three-month-old Swiss female mice were studied, and the vaginal smears were clas-

TABLE 2
β-GLUCURONIDASE IN THE MOUSE UTERUS

	Number	Units/gm./hr.	Units/organ
Proestrus	8	2068 ± 256	137.6 ± 13.8
Estrus	8	1927 ± 160	177.1 ± 18.2
Metestrus	8	1737 ± 194	140.1 ± 13.6
Diestrus	11	1578 ± 118	123.9 ± 12.5

sified as proestrus, estrus, metestrus, and diestrus. Glucuronidase activity (units/gm./hr.) was lower during diestrus than during other phases of the cycle, but only the differences between the enzyme concentrations at proestrus and diestrus approach significnace. Total uterine glucuronidase activity was maximal during estrus (TABLE 2).

Influence of Thiouracil and Chorionic Gonadotropin on the Rat Uterus

The influence of hypothyroidism on the biochemical composition of the uterus, and the response of this organ when gonadotropin is administered to the hypothyroid animal, have had little consideration. Thyroidectomy decreases uterine water and reduces the response to estrogen.[36] Recently, Glasser[37] has studied uterine composition in euthyroid and hypothyroid rats following deciduoma formation. Uterine glycogen increased when deciduomata were formed in normal rats, but no change in uterine glycogen was observed in thiouracil-fed rats, despite the trauma of deciduomata.

Lederer and Meersseman[38] fed propylthiouracil to immature rats for 22 days and noted a lesser stimulation of the uterus following chorionic gonadotropin in hypothyroid than in euthyroid rats. However, ovarian sensitivity to gonadotropin is enhanced by hypothyroidism, and ovarian cysts may form.[39] The infertility associated with ovarian cysts and the alterations in cycles may be reflected in uterine composition, suggesting the need for further study.

To study the influence of hypothyroidism on uterine composition, immature female rats 30 to 35 days of age were used. Hypothyroidism was induced by feeding 0.5 per cent thiouracil in a purified diet containing 20 per cent casein and 27 per cent fat for 30 days. Uterine weight increase was retarded by thiouracil feeding in 5 of the 7 groups of animals used. Tissue to be used for nitrogen determination was dried to constant weight, and water content was determined. Uterine water content was not influenced by thiouracil feeding, and uterine wet:dry weight ratios were unaltered. Uterine protein concentration was slightly lower in hypothyroid than in euthyroid rats.

The hypothyroid state did not influence glycogen, lactic acid, or cholesterol concentrations. Furthermore, ascorbic acid and β-glucuronidase concentrations were not altered; the smaller uteri of the thiouracil-fed rats had less total ascorbic acid and β-glucuronidase. Alkaline phosphatase concentration was increased by thirty days of thiouracil feeding, whereas the enzyme concentration decreased slightly in euthyroid rats when compared with initial controls. Thus, uteri from hypothyroid rats had significantly greater concentrations of alkaline phosphatase than did those from euthyroid rats of the same age (TABLE 3). Thiouracil feeding to rats for one month during the second month of life had essentially no effect on uterine composition except to increase alkaline phosphatase concentration.

Immature female rats fed thiouracil for 30 days were injected with 10 I.U. of chorionic gonadotropin daily for the last 20 days. Ovarian weight was threefold greater than that observed in similarly injected euthyroid rats. More follicles were present in the stimulated ovaries from hypothyroid rats. Despite hypothyroidism, uterine weight was not significantly different in the chorionic gonadotropin-treated animals. Uterine water content and protein concentrations were lower in gonadotropin-treated euthyroid rats than in hypothyroid rats, and these values were lower than those observed for normal controls of the same age.

Alkaline phosphatase concentration was decreased significantly by chorionic gonadotropin in euthyroid and hypothyroid rats in relationship to their respective nontreated controls. Furthermore, the greater enzyme concentration of the uterus from hypothyroid rats was maintained despite the effect of gonadotropin treatment. Uterine weight of the hypothyroid gonadotropin-treated rat was such that total alkaline phosphatase was greatest in this group (TABLE 3).

β-Glucuronidase concentration in the uterus was increased in rats treated with chorionic gonadotropin, whereas cholesterol concentration was decreased. However, the gonadotropin treatment did not decrease cholesterol concentration below that induced by thiouracil alone. Uterine ascorbic acid concentration did not respond despite the stimulating action of gonadotropin on the ovaries.

The increase in uterine weight that followed chorionic hormone administration to euthyroid rats did not increase glycogen concentration. However, a significant increase in uterine glycogen concentration was induced by gonadotropin administration in hypothyroid rats (TABLE 3.) Uterine lactic acid concentration was not influenced by thiouracil or gonadotropin treatment, but total lactic acid content of the uterus was increased with growth of the organ.

Administration of chorionic gonadotropin for twenty days induces heavy luteinization in the ovary. Associated with the ovarian changes, one finds a slight decrease in uterine water and protein concentration and the lack of change in glycogen and ascorbic acid that simulates the action of progesterone on the immature mouse uterus. While the increase in β-glucuronidase might suggest the action of estrogen, this could also be invoked by progesterone. The decrease in alkaline phosphatase induced by chorionic gonadotropin is duplicated by castration, but remains unresolved in our studies.

Hypothyroidism modified the ovarian response to chorionic gonadotropin. However, the decrease in alkaline phosphatase concentration, the increase in β-glucuronidase concentration and total lactic acid, and the absence of a change in ascorbic acid all simulated, in general, the response of the euthyroid rat to gonadotropin. Some differences were recorded for uterine water, and protein concentrations were not decreased in hypothyroid rats by gonadotropin treat-

TABLE 3

INFLUENCE OF THIOURACIL AND CHORIONIC GONADOTROPIN ON THE RAT UTERUS

	Control	Thiouracil	Chorionic gonadotropin	Thiouracil + C.G.
No. rats	7	6	8	5
Uterine wt. (mg.)	217	131	233	295
Dry wt.	39	24	51	53
Water (%)	81.7	81.5	78.2	81.9
Protein (%)	78.2	75.2	73.3	76.1
Alkaline phosphatase				
No. rats	9	6	13	15
Uterine wt. (mg.)	163	54	161	247
μg./mg./hr.	1.73 ± 0.3	2.94 ± 0.6	0.92 ± 0.2	1.70 ± 0.3
Total	233 ± 33	140 ± 20	141 ± 30	431 ± 68
β-Glucuronidase				
No. rats	9	7	13	12
Uterine wt. (mg.)	163	57	162	194
Units/gm./hr.	2653 ± 185	2758 ± 549	4295 ± 380	5806 ± 710
Total	621 ± 110	179 ± 55	620 ± 109	600 ± 59
Ascorbic acid				
No. rats	8	7	8	12
Uterine wt. (mg.)	163	97	223	139
μg./mg.	0.19 ± 0.08	0.14 ± 0.05	0.19 ± 0.07	0.16 ± 0.07
Total	30.4	13.6	41.6	23.5
Glycogen				
No. rats	7	7	10	9
Uterine wt. (mg.)	39	55	161	215
Per cent	0.69 ± 0.06	0.89 ± 0.02	0.55 ± 0.07	1.35 ± 0.18
Total	0.27 ± 0.05	0.40 ± 0.05	1.37 ± 0.18	2.86 ± 0.48
Lactic acid				
No. rats	10	10	10	10
Uterine wt. (mg.)	210	91	210	341
μg./mg.	1.8 ± 0.3	1.4 ± 0.2	1.1 ± 0.3	1.3 ± 0.2
Total	137.4 ± 8.2	125.2 ± 5.8	230.8 ± 7.9	418.2 ± 27.4
Cholesterol				
No. rats	6	11	8	9
Uterine wt. (mg.)	43	83	171	215
Free per cent	0.30 ± 0.05	0.24 ± 0.02	0.20 ± 0.01	0.21 ± 0.01
Total	143	218	371	435

ment, and a significant increase in glycogen was recorded. The increases in glycogen and β-glucuronidase might well be responses to estrogen or androgen, but the associated decrease in alkaline phosphatase is in conflict with this pattern. Undoubtedly further information from the study of steroid hormone combinations in euthyroid and hypothyroid states will be required before we can evaluate the current results more adequately.

Summary

The 72-hour response of the immature mouse uterus to estradiol dipropionate, estosterone propionate, and progesterone was studied. Water, protein, lipid,

alkaline phosphatase, β-glucuronidase, ascorbic acid, cholesterol, and glycogen were measured; estradiol influenced all components except cholesterol. Testosterone propionate failed to alter alkaline phosphatase and cholesterol. Progesterone had no effect on lipid.

During the estrous cycle, β-glucuronidase exhibited only minor variations. Administration of chorionic gonadotropin for 20 days invoked changes in the rat uterus that, except for a decrease in alkaline phosphatase, simulated progesterone administration. Hypothyroidism modified the uterine response to chorionic gonadotropin as to water, protein, and glycogen concentrations.

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STEROID INFLUENCES ON THE LACTIC DEHYDROGENASE-DPNH OXIDASE SYSTEM OF THE RAT UTERUS*

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It is the purpose of this paper to describe the effects of several steroid hormones upon two enzymes in the uterus of the rat. The material presented will be in part from material previously published and largely from unpublished findings. The data to be presented were gathered in two laboratories: at the Biological Laboratories, Harvard University, Cambridge, Mass., and at the University of Oklahoma School of Medicine. The findings represent collaborative work with Joseph T. Velardo and Frederick L. Hisaw at the former laboratories and with Phillip J. Campbell and Robert G. Schoenfeld at the latter. The author presents this paper as a representative spokesman for both groups.

We have chosen to study steroid effects upon lactic dehydrogenase (LDH) and upon reduced diphosphopyridine nucleotide (DPNH) oxidase,† since the two enzymes, as a system, control the reversible oxidation and reduction of lactate and pyruvate at the end of the glycolytic sequence of reactions, and because the latter enzyme as a hydrogen and electron acceptor from reduced diphosphopyridine nucleotide (DPNH) is an essential mediator for several DPN-linked dehydrogenation reactions. Steroid effects upon the activity level of these enzymes thus could have an important directing influence upon uterine metabolism. Our studies have demonstrated that this system in the rat uterus is very sensitive in its activity level to changes in the hormonal environment.

Effect of Estrogens

Data presented earlier by our group in 1954 show that during the estrous cycle, rat uterine LDH and DPNH oxidase activities are highest in proestrus and early estrus and lowest in diestrus.[1] The approach throughout all the steroid experiments to be discussed was to use 100-day-old rats from the Harvard colony. These were ovariectomized one week prior to steroid treatment. Preliminary experiments indicated that this was sufficient time for the enzyme level to fall to approximately half the estrous value and to or below the diestrous value. Ovariectomized animals allowed to go beyond two weeks required increased estrogen dosages to re-establish the original values. The-

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† The term DPNH oxidase as used here is a nonspecific term indicating the enzymes or enzyme systems that reoxidize DPNH in the presence of neotetrazolium chloride. As yet a precise characterization of the possible enymes involved has not been made. We therefore feel that a more definite terminology, for example diaphorase, a term applied to well-characterized flavin enzymes in heart and liver, is not justified. Alternatively, DPN-NTZ reductase, while precise, does not gain in physiological significance.

enzyme levels after one week of castration were unaffected by the estrous state at the time of ovariectomy. Animals received estrogens in 0.1 ml. of sesame oil administered subcutaneously once daily. When two steroids were administered, they were injected at separate sites. At the end of the experimental time, the rats were sacrificed and the uteri rapidly excised, freed of fat, weighed, homogenized, and assayed for enzyme activity. The enzyme methods have been previously described by Bever *et al.*[2] in 1956. Briefly summarized, the uterine homogenates were assayed in a system containing lactate as substrate, DPN as coenzyme, potassium cyanide as the pyruvate trapping agent, and neotetrazolium chloride (NTZ) as acceptor dye. When LDH was to be measured specifically, excess purified heart diaphorase was added to make the homogenate LDH limiting. Conversely, when DPNH oxidase was specifically measured, purified muscle LDH was added to insure DPNH oxidase to be limiting. In all cases with rat uterus, irrespective of estrous stage or hormone treatment, the endogenous DPNH oxidase was found to be the rate-limiting enzyme in the two-enzyme system. The enzyme unit for both enzymes as measured in this test is the micrograms of NTZ reduced per 30 minutes. The specific activity, unless otherwise indicated, is the units per milligram dry weight of tissue. Specific activity measured on this basis was found to be as valid and sensitive as expressing the activity in terms of nitrogen for uterine enzymes.

The effect of estrogen administration upon the enzyme system is illustrated in FIGURE 1. This shows the level of activity at various times up to 15 days with constant daily administration of estradiol, estriol, and combination

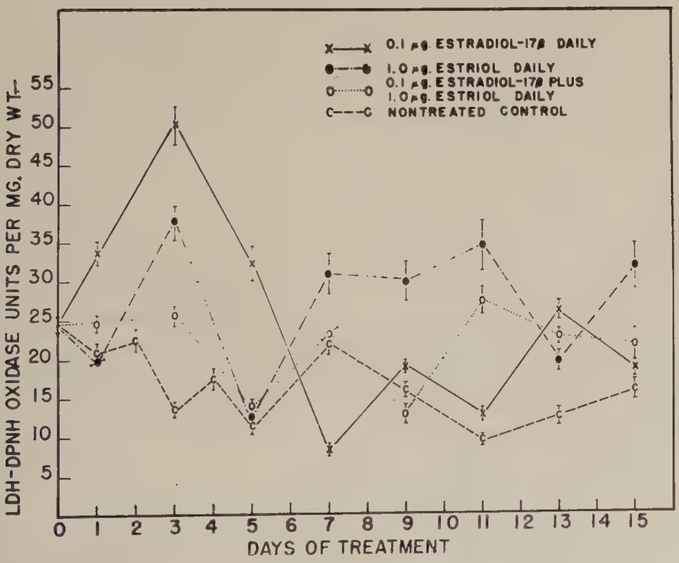

FIGURE 1. The effect of estradiol and estriol on ovariectomized rat uterine LDH-DPNH oxidase system. Each point represents the average from 12 to 18 determinations from 4 to 6 uteri. Standard error of the mean for each point is indicated by vertical lines. Prepared from data of Bever *et al.*[2]

dosages. In this experiment no attempt was made to separate the system into its two component activities. In view of later work mentioned above we are confident that the data represent a measure of the DPNH oxidase activity.

The data indicate the enzymatic activity levels reached with daily doses of 0.1 μg. estradiol, 1.0 μg. estriol, and the two estrogens in combination. Three things were of particular interest. One was the rapid rise of enzymatic activity achieved with either estradiol or estriol in the first few days of the period. The second was that, unlike the uterine fresh weight or dry weight that reached constant levels with each dosage and maintained these levels as demonstrated in 1954 by Hisaw *et al.*,[3] the maximum enzyme activity reached with each type of estrogen is not maintained. It is lost rapidly and reaches a minimal value after a few days, which is at or below the untreated control activity. This occurs even though continued daily administration of estrogen has been received. The third point of interest was that under the combined estrogen treatment the activity level was less than that of either estradiol or estriol alone.

When each enzyme was studied separately with increasing amounts of the three estrogens—estradiol, estrone, and estriol—using a three-day assay period, the data of FIGURES 2 and 3 were obtained. FIGURE 2 indicates that under these conditions estriol is capable of elevating uterine LDH to a higher level than estradiol or estrone and that, at lower dosages, estradiol is more efficacious than estrone in this respect. FIGURE 3 shows that estradiol and estrone promote the DPNH oxidase response of the system to a greater extent than does

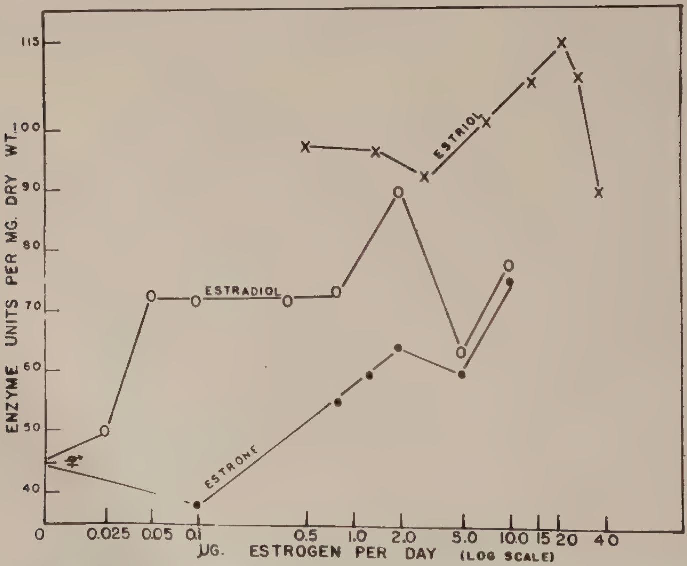

FIGURE 2. Effect of 3 estrogens on uterine LDH after 3 days of daily administration. Each point represents the average of 4 to 5 uterine horns distributed in 3 separately prepared homogenates. Prepared from data of Bever *et al.*[2]

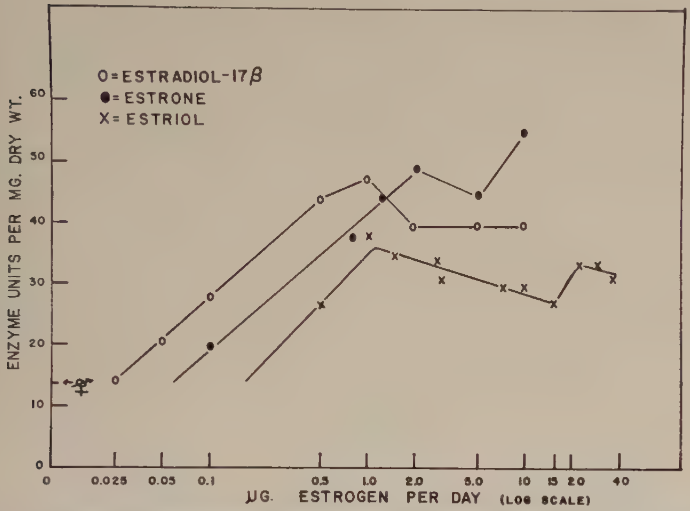

FIGURE 3. Effect of 3 estrogens on uterine DPNH oxidase after 3 days of daily administration. Each point represents the average activity of 4 to 5 uterine horns distributed in 3 separately prepared homogenates. Prepared from data of Bever *et al.*[2]

estriol. At dosages up to 1.0 μg. daily estradiol is the more potent, while at larger dosages estrone produces higher enzyme levels.

While the data are not presented here, an added comment may be made of our observations with diethylstilbestrol. This compound produced an effect on both LDH and DPNH oxidase that closely paralleled estrone in its effect at the same dosages. In combined studies with estradiol, there was no indication of interference between the two steroids since the resulting activities were additive. Such was also the case with estrone and estradiol given in combined dosages.

Effect of Progesterone

Progesterone was administered in the same manner as the estrogens in a dose range between 0.05 mg. and 1.0 mg.[4] It was given alone to some groups of ovariectomized animals and given simultaneously with 0.1 μg. estradiol to other groups. The activity levels of the two uterine enzymes were determined after 3 days of administration. The results are graphically presented in FIGURES 4 to 7. The effect of progesterone is shown to be different on the 2 enzymes. Progesterone alone (FIGURES 4 and 6) increases LDH activity significantly over the nontreated control group to as much as 230 per cent (0.5 mg. daily). On the other hand, progesterone alone does not augment DPNH oxidase (FIGURES 5 and 6). In fact, up to 0.5 mg. daily, the lower dosages mildly decrease the activity to values below those of the nontreated ovariectomized control group.

The inhibitory property of progesterone upon DPNH oxidase is more strongly illustrated when progesterone is given in combination with estradiol

(FIGURES 5 and 7). The DPNH oxidase level produced by 0.1 μg. estradiol alone is effectively reduced by increasing progesterone dosages to 47 per cent inhibition at 0.25 mg. daily. This level is slightly below the starting non-treated base level. The LDH activity of the lowest dosages of progesterone

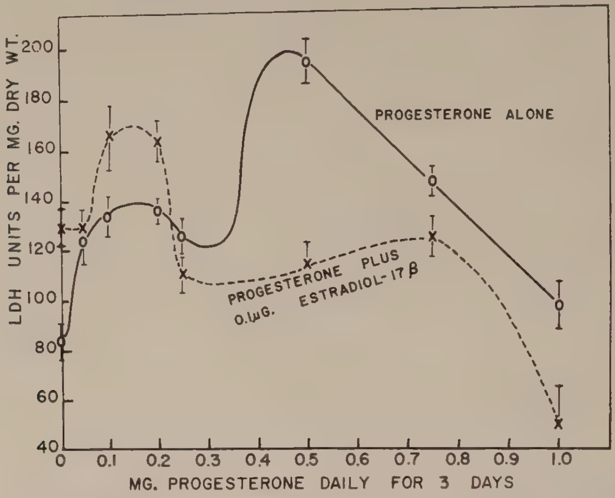

FIGURE 4. The effect of progesterone and of progesterone plus estradiol-17β after 3 days of daily administration on uterine LDH. Each point represents the mean value of 8 to 12 animals in 4 to 6 determinations using pooled uteri from 2 animals in each homogenate. The vertical lines represent standard error of the mean for each point.

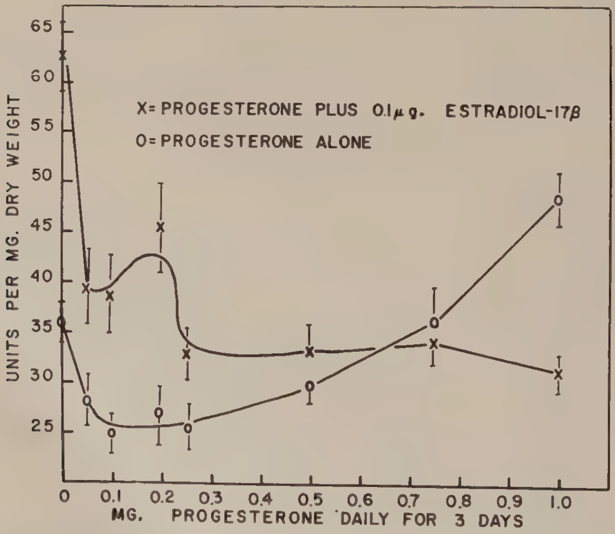

FIGURE 5. The effect of progesterone and of progesterone plus estradiol-17β after 3 days of daily administration on uterine DPNH oxidase. Each point represents the mean value of 8 to 12 animals in 4 to 6 determinations using pooled uteri from 2 animals in each homogenate. The vertical lines represent standard error of the mean for each point.

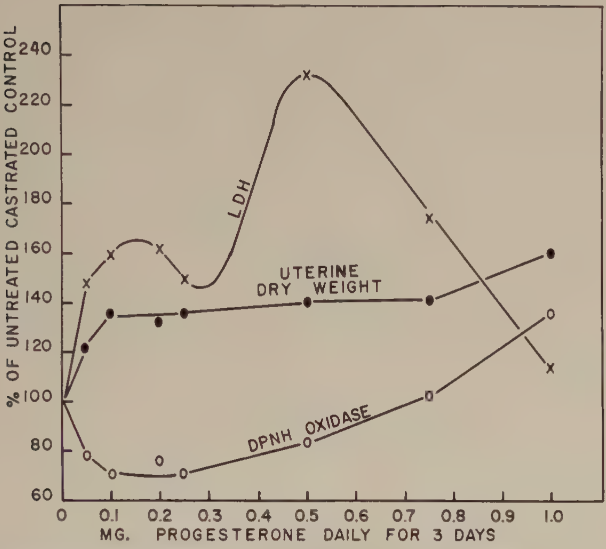

FIGURE 6. Influence of progesterone on rat uterine LDH-DPNH oxidase system. Calculated from data of FIGURES 4 and 5.

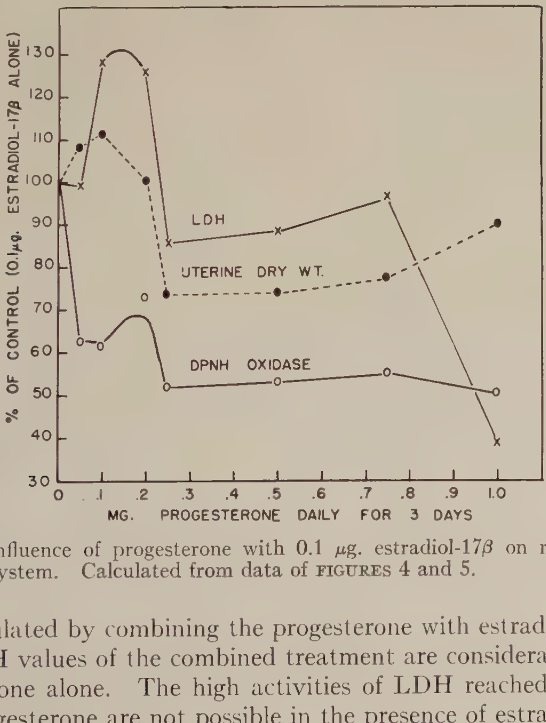

FIGURE 7. Influence of progesterone with 0.1 μg. estradiol-17β on rat uterine LDH-DPNH oxidase system. Calculated from data of FIGURES 4 and 5.

is mildly stimulated by combining the progesterone with estradiol. At higher levels the LDH values of the combined treatment are considerably lower than with progesterone alone. The high activities of LDH reached by the upper dosages of progesterone are not possible in the presence of estradiol.

It must appear then that in the rat uterus, estradiol and progesterone work in an opposing manner upon these two enzymes. Estradiol alone strongly stimulates the DPNH oxidase and moderately elevates LDH activity levels. Progesterone alone has a strong promotional effect upon LDH and mildly lowers the activity levels of DPNH oxidase to values below those of the untreated control. Progesterone prevents estradiol from exerting its stimulation upon DPNH oxidase and estradiol lowers the progesterone-induced response upon LDH. It would seem reasonable to assume that estradiol, by elevating both LDH and DPNH oxidase, has prepared an enzymatic mechanism by which lactate oxidation would be favored. By increasing LDH activity strongly and severely depressing DPNH oxidase (in the presence of estrogen) progesterone would appear to be creating an enzymatic machinery unfavorable to lactate oxidation, but favorable for pyruvate reduction to form lactate.

Effects of Desoxycorticosterone Acetate, Testosterone and Cortisone Acetate

Turning from progesterone, we next directed our attention to three different types of steroids. These were desoxycorticosterone acetate (DCA), testosterone, and cortisone acetate.[5] These were administered in the same experimental design that was used with progesterone—each steroid alone and each in combination with 0.1 μg. of estradiol. The results are presented in FIGURES 8 to 11. FIGURE 8 demonstrates the effect upon uterine LDH of each of the three steroids, administered alone, in relation to the effect of 0.1 μg. estradiol alone. Each of the three has a greater promotional effect than the estradiol

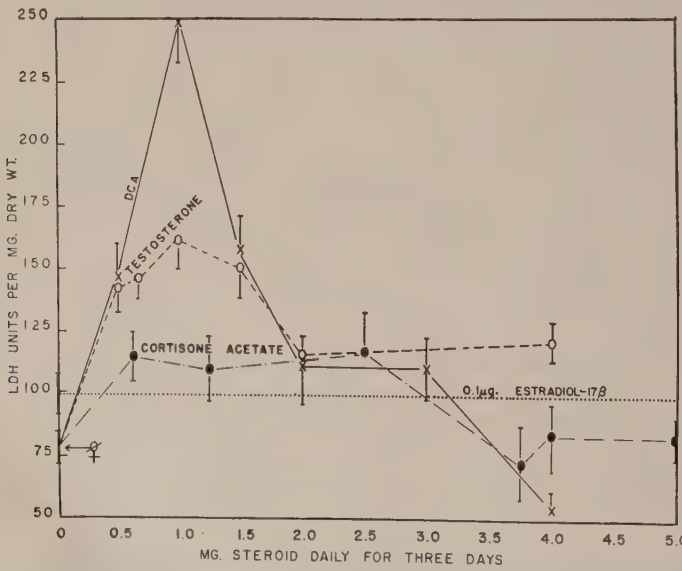

FIGURE 8. The effect of desoxycorticosterone acetate, testosterone, and cortisone acetate on uterine LDH of ovariectomized rats after 3 days of administration. Each point represents the mean value of 8 to 12 animals in 4 to 6 determinations. The vertical lines represent standard error of the mean for each point.

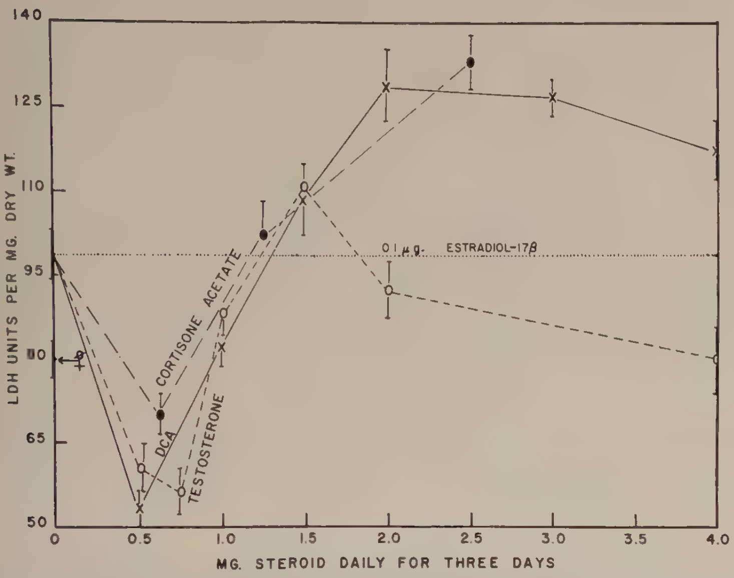

FIGURE 9. The effect of desoxycorticosterone acetate, testosterone, and cortisone acetate vhen each is administered with 0.1 μg. estradiol-17β upon uterine LDH of ovariectomized ats. The steroids were administered daily for 3 days. Each point represents the mean value of 8 to 12 animals in 4 to 6 determinations. The vertical lines represent standard error of the mean for each point.

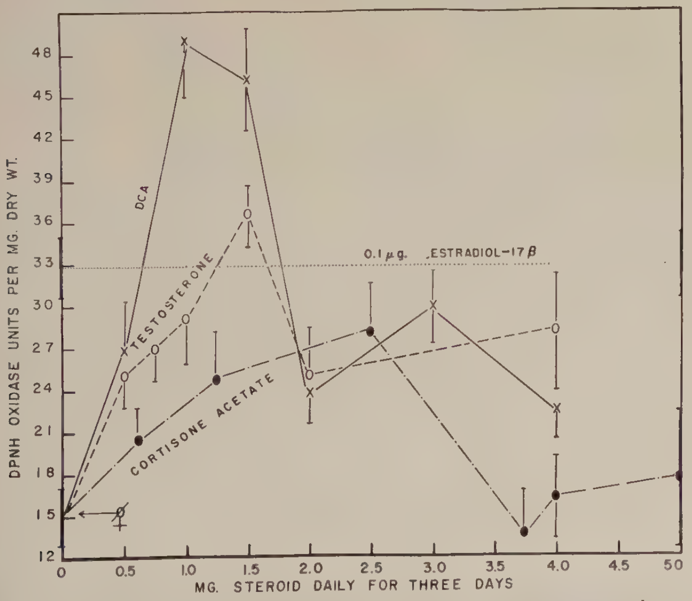

FIGURE 10. The effect of desoxycorticosterone acetate, testosterone, and cortisone ace-ate on uterine DPNH oxidase of ovariectomized rats after 3 days of administration. Each oint represents the mean value of 8 to 12 animals in 4 to 6 determinations. The vertical ines represent standard error of the mean for each point.

dosage throughout most of the steroid concentration range employed. DCA is particularly effective in its lower dosages in elevating LDH. DCA apparently can elevate LDH activity to levels as high as those reached under progesterone treatment, but requires twice the amount to do it. Testosterone has about one half the effectiveness of DCA in this respect, while cortisone acetate is even less effective. The ability of each of these steroids to promote LDH is severely interfered with if estradiol is also administered. FIGURE 9 shows the inhibited values reached in the lower dosage amounts when each steroid is administered with estradiol. The lowest LDH level produced by DCA and estradiol and the lowest level attained by testosterone and estradiol are nearly the same. However, the effects produced by these two combinations diverge in levels of steroid above 1.5 mg. steroid daily. At the upper steroid dosages DCA and cortisone acetate with estradiol generally elevate LDH to higher activity levels than did equivalent amounts of these steroids alone, while testosterone with estradiol at upper dosages produced lower LDH activities than did testosterone alone at equivalent concentrations.

The effect of these three steroids, alone, on DPNH oxidase was generally similar to that of the steroids on LDH (FIGURE 10). DCA was most effective in increasing DPNH oxidase, followed by testosterone and cortisone acetate. It is interesting in this respect to note that DCA increases both LDH and DPNH oxidase, while progesterone, which is structurally and metabolically

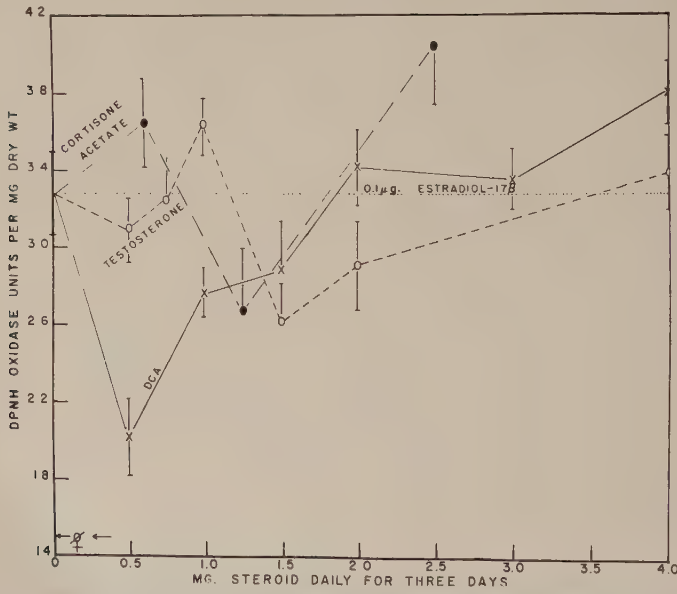

FIGURE 11. The effect of desoxycorticosterone acetate, testosterone, and cortisone acetate when each is administered with 0.1 μg. estradiol-17β upon uterine DPNH oxidase of ovariectomized rats. The steroids were administered daily for 3 days. Each point represents the mean value of 8 to 12 animals in 4 to 6 determinations. The vertical lines represent standard error of the mean for each point.

TABLE 1

EFFECT OF STEROIDS ON RAT UTERINE LDH-DPNH OXIDASE ACTIVITY

Steroid	Relative enzyme activities			
	Effect of steroid alone		Effect of steroid given with estradiol	
	LDH	DPNH oxidase	LDH	DPNH oxidase
(1) Estradiol-17β	+++	++++	—	—
(2) Estrone	++	+++	—	Additive effect
(3) Estriol	++++	++	?	Estriol inhib. estradiol — — — —
(4) Progesterone	+++++	— —	Estradiol inhib. prog. — — —	Prog. inhib. estradiol — — — —
(5) DCA	++++	+++	Estradiol inhib. DCA — — — —	DCA inhib. estradiol — — —
(6) Testosterone	++	++	Estradiol inhib. test. — — — —	Test. inhib. estradiol — —
(7) Cortisone acetate	±	+	Estradiol inhib. cort. — —	No effect

elated, stimulates only LDH and is even mildly suppressive to DPNH oxidase vhen given alone. When given with estradiol simultaneously, however, the ffect of DCA and progesterone on DPNH oxidase are the same. Both act to lepress DPNH oxidase activity levels. FIGURE 11 illustrates this property f DCA. Testosterone and cortisone acetate do not appear to have any trong effect in modifying the action of estradiol in either direction.

The comparative effects of the steroids upon the two enzymes, alone and vith estradiol, are qualitatively summarized in TABLE 1.

nfluence of Adrenalectomy and Hypophysectomy upon Estradiol-Induced Changes in Uterine DPNH Oxidase

When 0.1 μg. estradiol is administered daily as in the experiment shown in IGURE 1, the immediate rise in DPNH oxidase is not maintained at an elevated evel, but declines rapidly. Since the fresh weight and dry weight remain at an levated level throughout the period,[3] the phenomenon appears to be unrelated o the general protein response induced by estradiol administration. Our indings with progesterone and DCA indicated the suppressing effect these teroids displayed upon estrogen-induced changes in DPNH oxidase. An xperiment became imperative, therefore, to determine whether an adrenal or ituitary-adrenal secondary control might be operating to bring about the bserved decrease. If the adrenal, either as a direct result of injected estradiol r, secondarily, through pituitary stimulation, increased its output of inhibitory teroids, then adrenalectomy or hypophysectomy should remove the delayed nhibitory response to injected estradiol. The following experiments were ndertaken in collaboration with Phillip J. Campbell and Robert G. Schoeneld.[6]

For these experiments, we used 60-day-old rats (Holtzman), rather than

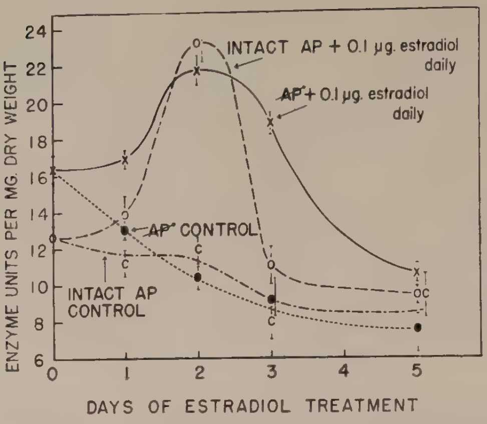

FIGURE 12. Effect of hypophysectomy on uterine DPNH oxidase response to estradiol-17β. Each point represents the mean value of 6 to 8 animals in 4 to 5 pooled uterine homogenates run in triplicate. Vertical lines represent standard error of the mean.

100-day-old rats, in order to facilitate the operational technique for hypophysectomy. All animals were ovariectomized 1 week before beginning estradiol administration. Hypophysectomy was performed on the fifth day after ovariectomy and adrenalectomy on the following day when combined operations were required. When adrenalectomy only was required, operation was on the fifth day after ovariectomy. Completeness of hypophysectomy was confirmed at autopsy and dubious animals discarded. Salt was made available in trays in the cages of all operated animals. Rats receiving estradiol received 0.1 μg. daily in 0.1 ml. sesame oil. Controls received only the oil.

This study was limited to DPNH oxidase responses since previous studies had indicated it to be the limiting rate step in the two-enzyme system and because LDH activity does not exhibit so pronounced a decline in activity on continued estradiol administration.

FIGURE 12 illustrates the DPNH oxidase response to estradiol of hypophysectomized and ovariectomized rats in relationship to ovariectomized rats with intact pituitaries. While the study indicates a slightly higher beginning level for the hypophysectomized animals, neither the ability of estradiol to induce a response nor the resulting loss of activity is lost by hypophysectomy. The percentage increase is lessened and the rate of loss of activity is slowed somewhat by hypophysectomy. The earlier time at which maximum activity of the controls (nonoperated) is reached as compared to the data in FIGURE 1 is believed to be due to the smaller size of the animals, resulting in a larger effective concentration of estrogen. One may conclude that an intact hypophysis is not necessary for the stimulation by estradiol, nor is it necessary to mediate the delayed loss phenomenon.

In a following experiment some animals were adrenalectomized and others were both adrenalectomized and hypophysectomized. The effect of both these operations is illustrated in FIGURE 13. Again a rise with estradiol treat-

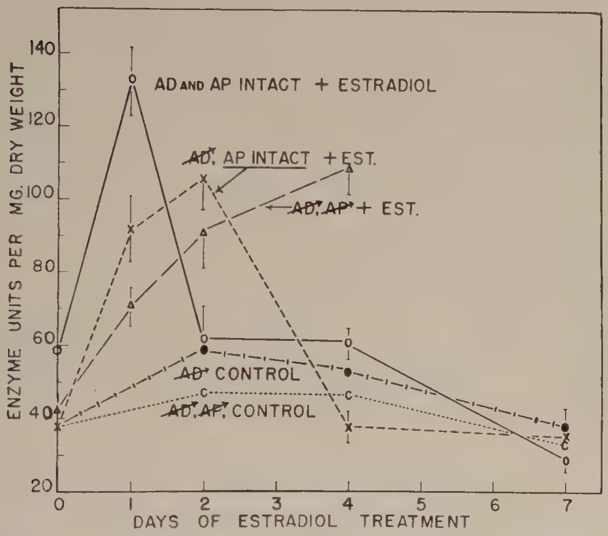

FIGURE 13. Effect of adrenalectomy and hypophysectomy on uterine DPNH oxidase esponse to estradiol in ovariectomized rats. Each point represents the mean value of 7 to animals in 4 to 5 pooled uterine homogenates run in triplicate. Vertical lines represent tandard error of the mean.

nent in adrenalectomized animals is observed. The maximum at day 2 is less han the maximum at day 1 of the intact control group receiving estradiol. Iowever, the actual maximum for the former may have occurred between ays 1 and 2. Adrenalectomy does not prevent a drop in activity, but it does low it down, since a minimum for the group is not reached until day 4. The ffect of doubly operating on the animals was not evaluable in this experiment n that the group prepared for day 7 did not survive. Consequently another xperiment to ascertain the effect of adrenalectomy and hypophysectomy was onducted. The results are shown in FIGURE 14. On a 10-day experiment nimals that were doubly operated show a maximal rise between 3 and 4 days, ollowed by a loss of activity.

Adrenalectomy or combined adrenalectomy and hypophysectomy under hese conditions, then, does not prevent estrogen-induced responses, nor does he loss of these glands prevent the subsequent collapse of activity. The terine response to estradiol in ovariectomized rats in the specific elevation of)PNH oxidase is not primarily dependent upon either an intact pituitary or he intact adrenal gland. It may also be concluded that the inability to main- ain an elevated enzyme activity and the development of refractoriness are ot dependent upon the presence of either of these glands, although the adrenal nay contribute to the phenomenon.

Our data would indicate that while the loss of either pituitary or adrenals oes not fundamentally change the character of the estradiol-induced response, he loss of either may operate to slow down the response. However, the

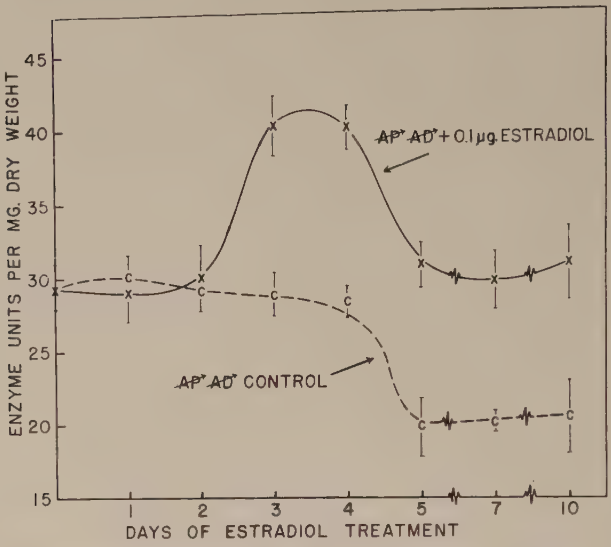

FIGURE 14. Effect of adrenalectomy and hypophysectomy on uterine DPNH oxidase response to estradiol in ovariectomized rats. Each point represents the mean value of 6 to 8 animals in 4 to 5 pooled uterine homogenates run in triplicate. Vertical lines represent standard error of the mean.

general metabolic state of the animals following operation would also explain the quantitative differences.

An alternative explanation for the development of the "delayed-loss" characteristic is suggested by the effects of estriol when administered with estradiol. This hypothesis would suggest that the build up of products of estradiol metabolism is responsible for the subsequent loss in activity. We are currently investigating this possibility, but definitive data are still lacking.

In Vitro Stimulation of LDH and DPNH Oxidase

In collaboration with Schoenfeld we have made many attempts to demonstrate an *in vitro* effect of added estradiol and progesterone to uterine strips, homogenates, and slices. The steroids have been introduced in many various ways, in suspensions, oil emulsions, in solvents such as ethanol, butyl alcohol, propylene, and ethylene glycol, and in protein-containing systems, as in bovine serum albumin, rat serum, and whole rat blood. Various time periods of incubation from thirty minutes to forty-eight hours have been employed. All attempts to demonstrate an augmentation of either LDH or DPNH oxidase have met with failure.

In view of the dramatic success of Claude Villee and his collaborators[7] in demonstrating *in vitro* stimulation of DPN-dependent uterine isocitric dehydrogenase in human term placenta,[7] our negative results lead only to the conclusion that the apparent increase in LDH and DPNH oxidase is due to a secondary effect following a primary action by estradiol elsewhere leading to a relatively specific protein synthesis. The elevation of LDH and DPNH oxidase by estradiol is believed to be part of the same effect that leads to elevated succinoxidase activity as originally observed in 1953 by M. A. Telfer[8] and confirmed by B. B. Longwell and A. E. Reif.[9] The observed changes in LDH and DPNH oxidase may be the result, then, of earlier changes brought about by estrogens in the nucleic acid components of the uterus as demonstrated in the work of Gerald C. Mueller.[10] If this is true, it is possible that these or other enzymes may be used as indicators in tracing the role of estradiol in modifying protein synthesis from the nucleic acid changes. While such a mechanism is lacking, the loss of enzyme activity after an initial rise may be tentatively interpreted as an indication of an estrogen-directed control of protein synthesis organizing the synthesis of new protein more important than DPNH oxidase to uterine function as growth proceeds. Such a view would be strengthened by finding an enzyme rising in activity as DPNH oxidase loses activity.

Relationships of DPNH Oxidase and DPNH-Cytochrome c Reductase*

In the studies presented thus far, concentration of interest has centered upon steroid influences on the enzyme activity of DPNH oxidase. A natural extension of this interest has been to study the steroid effect on uterine tissue to reduce ferrocytochrome *c* with DPNH as the electron donor. More specifically of interest is whether the enzymatic activity identified with the observed ability to reduce NTZ is associated with the same enzymes or parts of the same system utilized in reducing cytochrome *c*. Particularly of interest is the question whether the elevation in activity brought about by estradiol in the case of NTZ reduction holds true for cytochrome *c* reduction. Studies by F. M. Huennekens and his co-workers[11] have indicated that in heart tissue there may be a pathway open for DPNH oxidation by mechanisms not involving cytochrome *c* and cytochrome oxidase.

Effect of Estradiol

The experiment to be described was designed to study the comparative effect of estradiol upon DPNH oxidase and DPNH-cytochrome *c* reductase.

Method

Holtzman rats approximately 90 days of age were ovariectomized 2 weeks before estradiol administration began. Groups of 6 to 8 rats each were then injected with varying amounts of estradiol in 0.1 ml. sesame oil once daily for 3 days. One group of 7 rats used as nontreated controls were injected on 3 successive days with 0.1 ml. sesame oil. Twenty-four hours after the last injection the rats were sacrificed. The uteri were freed of fat and weighed. Two uteri were utilized for preparing each homogenate. A 4 per cent fresh-weight homogenate was prepared in 0.05 M Tris buffer (2-amino-2-hydroxymethyl-1,3-propanediol), *p*H 7.8. Aliquots of the homogenate were used to

* Studies in this section were conducted with the collaboration of Robert G. Schoenfeld.

determine DPNH oxidase activity, cytochrome *c* reductase activity, desoxyribonucleic acid (DNA) concentration, and nitrogen.

DPNH oxidase was determined by the previously published method[2] with three minor modifications: (1) only 0.25 ml. and 0.5 ml. of homogenate were used as enzyme source, with 1.0 ml. of 0.067 M phosphate buffer, *p*H 8.2, added to each incubation tube; (2) the time of incubation was 15 minutes rather than the previous 30; (3) after the trichloracetic acid precipitation step, the residue after centrifugation was washed with 1.0 ml. of 0.1 N HCl to remove interfering substances before the reduced dye was extracted with cold acetone for colorimetric measurement at 505 mμ.

DPNH-cytochrome *c* reductase was measured spectrophotometrically by following the reduction of ferrocytochrome *c* at 550 mμ with DPNH as substrate. Contents and concentrations of test cell components are given with FIGURE 15. This assay represents a modification of the procedure of Henry Mahler,[12] as adapted for uterine tissue. No potassium cyanide is required for assay in uterine tissue since a very low cytochrome oxidase activity is present,

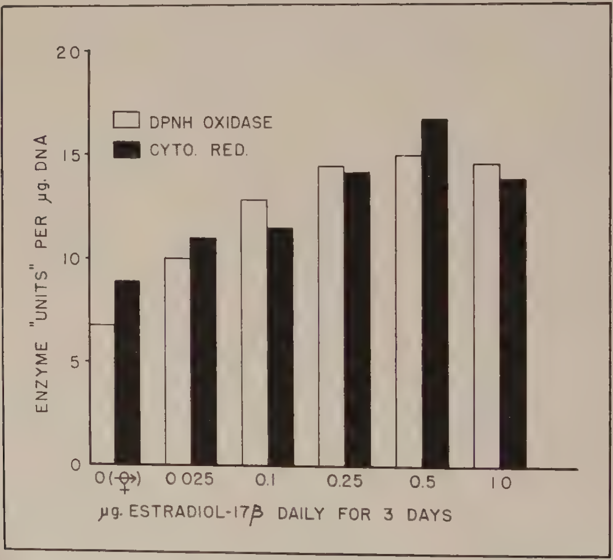

FIGURE 15. The comparative effects of estradiol-17β on DPNH oxidase and DPNH-cytochrome *c* reductase activities after 3 days of daily administration. Each bar represents the average activity of 5 to 7 animals in 3 separately prepared homogenates.

DPNH-cytochrome *c* reductase assay: optical cell contents. Test: DPNH, 0.2 ml. (1.5×10^{-3} M) in 0.1 M Tris buffer, *p*H 8.0; ferrocytochrome *c*, 1.5 ml. (2.0×10^{-4} M in 0.79 per cent sodium chloride); Tris buffer, 1.2 ml., 0.1 M, *p*H 8.0; homogenate, 0.1 ml, 4 per cent. Control: no DPNH added; 1.4 ml. Tris buffer; otherwise same as test cell. Homogenate added last at zero time and cells inverted twice. Readings made each 15 sec. for 3 min.

The unit of activity is 1000 × optical density change per minute for first 2 minutes. Specific activity = units of activity per μg. of DNA. DPNH oxidase specific activity = μg. NTZ reduced in 15 minutes per μg. DNA sufficient to alter detectably ferricytochrome *c* concentration in a three-inute assay.

DNA determinations were made on the homogenate immediately after omogenization by the method of J. M. Webb and H. B. Levy, employing -nitrophenyl hydrazine as color reagent.[13] Nitrogen determinations were ade by microkjeldahl digestion and direct nesslerization.

Results

The data presented in FIGURE 15 clearly indicate that the effect of estradiol pon DPNH oxidase as previously shown in FIGURE 3 is also reflected equally corresponding changes in the DPNH-cytochrome *c* reductase activity. he two activities are shown here upon a DNA basis in order to illustrate the nzymatic changes more nearly on a "per cell" basis rather than on total rotein change. The same data calculated on a nitrogen basis show the same ype of change with each enzymatic activity.

DISTRIBUTION OF ACTIVITIES IN CENTRIFUGAL FRACTIONS

Three different experiments with 2 uteri in each experiment were performed n rat uteri in the proestrus phase. These were homogenized in 0.25 M sucrose, nd fractions were obtained which were representative of tissue components sedinentable at (1) 600 relative centrifugal force (RCF) for 15 minutes; (2) 10,000 CF for 30 minutes; (3) 144,000 RCF for 1 hour; and (4) supernatant from raction C. Because of the heterogeneity of uterine tissue, these fractions annot be represented as nuclei, mitochondria, microsomes, or supernatant ractions, respectively, in this case. However, it is likely that a rough fracionation into approximately these fractions did occur, as judged by the disribution of enzyme activity as shown in Table 2. All of the LDH activity vas found in Fraction D. The DPNH oxidase and DPNH-cytochrome *c* eductase activity were both found in Fractions B and C. They were distribted approximately 40 per cent in Fraction B and 60 per cent in Fraction C. These findings would indicate that the two activities may be part of the same ntracellular fractions and further illustrate the close relationships between the wo activities.

TABLE 2

DISTRIBUTION OF ENZYMATIC ACTIVITIES IN CENTRIFUGAL FRACTIONS

	Per Cent of total activity		
Fraction	LDH	DPNH oxidase	DPNH-cyt. *c* red.
. 600 RCF	0	<1	<1
. 10,000 RCF	0	38	40.5
. 144,000 RCF	0	61	58.0
. Supernatant	100	<1	<1

INHIBITOR STUDIES

Experiments with various substances that have been shown to inhibit cytochrome *c* reduction in heart tissue were studied on the two enzymatic activities in uterine homogenates. Brief summaries of these follow here, and the data will be published more completely elsewhere. All inhibition experiments were done with normal rats in the proestrus stage of the estrous cycle. The effect of each inhibitor upon the two enzyme activities was studied at equal inhibitor concentration and equal inhibitor to tissue ratios.

Sodium Amytal. This substance inhibited neither DPNH oxidase nor DPNH-cytochrome *c* reductase in concentrations up to 2.8×10^{-4} M in uterine homogenates, and showed no enzyme inhibitory power on any differential centrifugal fractions of the homogenate. This lack of inhibition in uterine tissue is surprising in view of the 1957 report of R. W. Estabrook[14] on the inhibition shown by Sodium Amytal on cytochrome *c* reductase from heart muscle, and the 1956 paper by B. Chance[15] reporting inhibition in phosphorylating liver mitochondria. The inhibition in the former case was reported to be on the reduced flavoprotein, preventing reoxidation; in the latter report, it inhibited the reduction of flavoprotein by DPNH. The insensitivity of uterine DPNH oxidase and DPNH-cytochrome *c* reductase should be strongly suggestive of interrelationship through a common mediator.

*Antimycin A.** This antibiotic substance did not inhibit DPNH oxidase at any concentration studied in homogenates or any centrifugal fraction. It did not inhibit uterine homogenate DPNH-cytochrome *c* reductase at a concentration of inhibitor (4.75×10^{-6} M) and at inhibitor to protein ratio (690), which inhibited heart homogenate DPNH-cytochrome *c* reductase 55 per cent. At twice this concentration, the uterine homogenate enzyme system was inhibited 26 per cent. Mitochondrial fractions and microsomal fraction activity were equally inhibited to the extent of 25 per cent. The sensitivity of uterine tissue DPNH-cytochrome *c* reductase to antimycin A is approximately one quarter that of heart tissue enzyme. With this inhibitor in heart tissue the blockage was shown by Estabrook[13] to be at the cytochrome *b*, c_1, and *c* levels rather than at the flavoprotein level. If this is true in uterine tissue, it would indicate that DPNH oxidase measured by NTZ does not depend upon cytochrome *c*, or at least not an antimycin A-sensitive mediator.

α-Tocopheryl phosphate. The locus of action of α-tocopheryl phosphate inhibition in the succinoxidase system of heart tissue has been indicated to be above that of succinic dehydrogenase, probably at the ferrocytochrome *c*, preventing reduction (H. P. Jacobi *et al.*[16]). The results we obtained with uterine DPNH oxidase and DPNH-cytochrome *c* reductase indicate approximately equal inhibition of both activities (TABLE 3). α-Tocopherol and tocopheryl acetate are ineffective as either inhibitors or activators of these activities when added *in vitro*.

With these data available in respect to the relationships of the DPNH

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* The antimycin A used in these experiments was donated by F. M. Strong, Department of Biochemistry, University of Wisconsin, Madison, Wis.

TABLE 3

INHIBITION BY α-TOCOPHERYL PHOSPHATE

Final concentration of α-tocopheryl phosphate	Percentage of inhibition	
	DPNH oxidase	DPNH-cyt. *c* red.
1) 6.30×10^{-7} M	19	7
2) 3.15×10^{-6} M	38	32
3) 6.30×10^{-6} M	47	43
4) 1.26×10^{-5} M	54	52
5) 2.52×10^{-5} M	71	—

oxidase and DPNH-cytochrome *c* reductase activity, showing equal estradiol effects on each, the equal distribution in the centrifugal fractions, and the behavior with the inhibitors studied, we are now following the hypothesis that he portion of the enzyme or enzyme system responsible for DPNH oxidase hat is influenced by estrogens is measured equally, whether by NTZ or by cytochrome *c* reduction.

Summary

The results of studies of the effects of several steroids upon ovariectomized ats has been presented. These results seem to indicate that lactic dehydrogenase and portions of the enzyme system concerned with DPNH oxidation may be influenced by some of the steroids studied. Evidence is offered to show hat estradiol has the most potent stimulatory effect on DPNH oxidase, while progesterone is the most potent in effecting an increased LDH activity. Desoxycorticosterone acetate has an effectiveness very near that of progesterone upon LDH. Various mutual inhibitory influences were noted within the teroids studied. Progesterone, DCA, and estriol were shown to restrict estradiol promotion of DPNH oxidase. Estradiol prevented both progesterone and DCA from fully potentiating LDH activity.

Experimental evidence was presented to show that estradiol affects uterine DPNH oxidase, as measured by neotetrazolium reduction, and DPNH-cytochrome *c* reductase equally. Inhibitor studies show that the two activities espond similarly to inhibitors, and differential centrifugal studies indicate an qual distribution of the two activities within the centrifugal fractions.

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HISTOCHEMICAL OBSERVATIONS OF OXIDATIVE ENZYME SYSTEMS IN THE UTERUS AND VAGINA OF THE RAT

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The problems that beset investigators of uterine and vaginal growth and netabolism are many and varied. It has been and still is our conviction that ytochemical and histochemical studies particularly guided toward elucidating atterns of oxidative enzymatic activity in the uterus eventually will provide nformation necessary for a thorough understanding of quantitative biochemical nalyses. In the following studies it has been our intention to indicate that he separate histological structures of the uterus show discrete enzymatic esponses by such enzymes as the succinic dehydrogenase (SDH) and DPN-liaphorase (DPN) systems during the normal changing hormonal environment. n addition, each of these systems appears to have its own pattern of reactivity, ometimes distinct or otherwise overlapping with the other. Although precise nterpretations of these results are not yet possible, histochemical observations f these enzyme systems, which are associated intimately with the synthesis f high energy phosphate compounds (adenosine triphosphate), indicate those reas in the organ wherein the most probable effective hormonal stimulation of netabolic activity occurs.

MATERIALS AND METHODS

Over 100 female albino rats (Harvard strain) approximately 100 days of age vere used in this study. The animals were followed through 3 consecutive strous cycles by the vaginal-smear technique. During the fourth cycle they vere selected and sacrificed at the following stages: preproestrus, proestrus, strus, metestrus, 36-hour diestrus, and 60-hour diestrus. At least 10 animals vere studied for each stage; the results reported represent what is believed to e a typical reaction for the given periods of the cycle. Pieces of tissue were emoved from the mid-region of the vagina and uterus as quickly as possible nd were frozen immediately upon a block of dry ice. Tissues were stored in he frozen state until processed histochemically. For the demonstration of he SDH system, the technique of Rosa and Velardo (1954a) was used, vhile a substituted method employing sodium lactate as substrate and liphosphopyridine nucleotide as cofactor was employed to indicate the DPN-liaphorase system (DPN). These procedures involve the use of neotetrazolium NTZ, NT) which has the property of being reduced to a colored, insoluble liformazan presumably at the histological site of enzymatic activity. Cyto-ogical distribution of this intracellular precipitate is not believed to be adequate nd reliable for cytochemical interpretation. Farber *et al.* (1956a, 1956b) and ternberg *et al.* (1956) have indicated that, in all probability, similar procedures reveal a part of the system responsible for the oxidation of succinic acid, apparently a flavoprotein, while the oxidation of lactate is histochemically visualized as a result of the reduction of neotetrazolium by DPN-diaphorase.

Studies on the female target organs were carried out by us earlier, and preliminary reports have been presented elsewhere (Rosa and Velardo, 1953, 1954b, 1954c). It is significant to indicate that the methods used by us represent an earlier segment in the evolution of tetrazolium salts and their application to animal tissues for the localization of oxidative enzyme systems. Newer techniques have since appeared, the most recent of which include a new indicator dye, nitro-BT (Nachlas *et al.*, 1957). Special properties of this compound make it particularly favorable for use, not only as a histochemical, but also as a cytochemical tool for the demonstration of these enzymes. The superiority of nitro-BT methods must be acknowledged; preliminary studies by us have shown that with its use more citical enzyme patterns are obtained in uterine and vaginal structures. However, our results thus far with nitro-BT have shown a basic similarity in localization, regardless of intensity of reaction, thereby still validating our results at the histochemical level of study.

OBSERVATIONS AND RESULTS

Uterus

DPN-Diaphorase. Variations in the DPN activity in various regions of the normal, cyclical uterus are illustrated in FIGURES 1 to 5. A generally high activity was found in all components of the uterus during the "ballooned" or proestrous stage (FIGURE 1). The luminal epithelium reacted most intensely, while the glands showed a lesser but relatively strong reaction. Blood vessels (tunica media) paralleled the luminal epithelium in respect to the amount of precipitated diformazan. The stromal cells presented a staining picture commonly found in fibroblasts in connective tissue of the digestive tract (Rosa, 1954, 1955), but more diformazan seemed to be present within their cytoplasm. Clumps of diformazan apparently not associated with any cellular structures were found in the uterine stroma in areas close to the highly reactive structures described above. It is possible that this latter staining picture may have resulted from diffusion of some enzyme from higher regions of activity. The muscularis (circular and longitudinal layers) reacted moderately in most areas,

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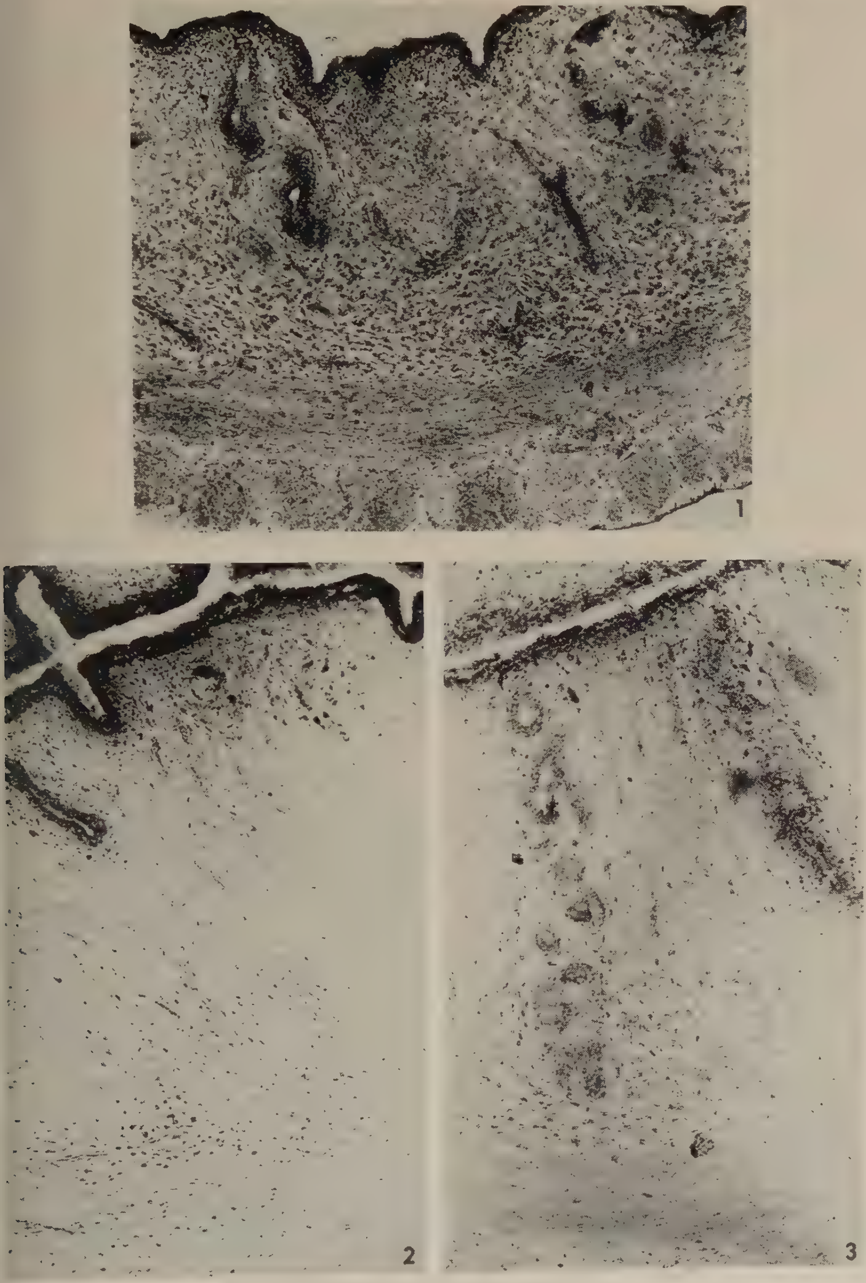
FIGURES 1 to 5 show the localization of uterine DPN activity, and FIGURES 6 to 11 demonstrate the distribution of SDH. The uterine musculature is oriented toward the lower portions of all photographs except FIGURE 11, where it is on the left. All illustrations of uterus represent transverse sections of this organ seen at a magnification of × 102.

FIGURE 1. Proestrus. This "ballooned" uterus shows intense DPN activity in the luminal and glandular epithelium. Blood vessels are also quite active ("Y"-shaped structure near the center toward the right-hand side). Muscle (circular and longitudinal) shows a moderate deposition of diformazan. Note the high activity of the stromal cells.

FIGURE 2. Estrus. Activity is highest in the luminal epithelium. Compared with proestrus, the glandular epithelium, stroma, and muscularis have lost significant amounts of activity.

FIGURE 3. Metestrus. Note the decided drop in the activity of the luminal and glandular epithelium. Activity in the muscle is quite low.



Figures 1 to 3

although some regions of slightly lower activity were observed in these structures. The proestrous uteri in these enzyme studies contained numerous "exogenous" cells within the stroma which, although completely unreactive for DPN, appear as dark bodies in the photograph. These unstained bodies, which show a considerable density to light, may possibly be mast cells.

The epithelium lining the uterine cavity presented a more intense reaction during estrus (FIGURE 2) than that of the preceding stage. Glands remained unchanged, but a very decided drop in activity was evident in the blood vessels, stroma, and muscularis. During metestrus, however, the uterine musculature appeared to regain some of its ability to reduce NTZ (FIGURE 3). There was some variation in stainability of the uterine glands at this point, and these structures, along with the luminal epithelium, showed considerable reduction in activity. The reaction of the stroma and blood vessels was quite similar to that found during estrus; these mostly retained this same degree of activity until the next proestrus.

The stage of 36-hour diestrus was characterized by a continued decline in DPN activity in the luminal and glandular epithelium (FIGURE 4). The muscular layers also stained more weakly than during metestrus.

There was an indication of decreased activity in the luminal epithelium of uteri taken at 60-hour diestrus (FIGURE 5). However, the muscularis continued to decline to a point where little or no reaction occurred. The glands remained unchanged.

Succinic dehydrogenase system. As was the case with DPN, the "ballooned" uteri of proestrus showed maximal reactions for SDH (FIGURES 6 to 11) and, in general, the staining of these uteri (FIGURE 6) resembled that of proestrous uteri stained for DPN. The exceptions to this were the almost complete lack of diformazan deposition within the stromal cells, and a low degree of staining in the blood vessels; both of these remained unchanged during the various stages of the estrous cycle. In some instances, glands were found also to contain more precipitate than those corresponding structures in DPN-stained proestrous uteri. At late proestrus a striking drop in the enzymatic activity of the muscularis occurred (FIGURE 7). Evidence of a slight drop in the SDH activity of the luminal epithelium also was found.

The reduced activity in the muscularis and glandular epithelium appeared to be a prelude to the generally low stainability of these uterine components

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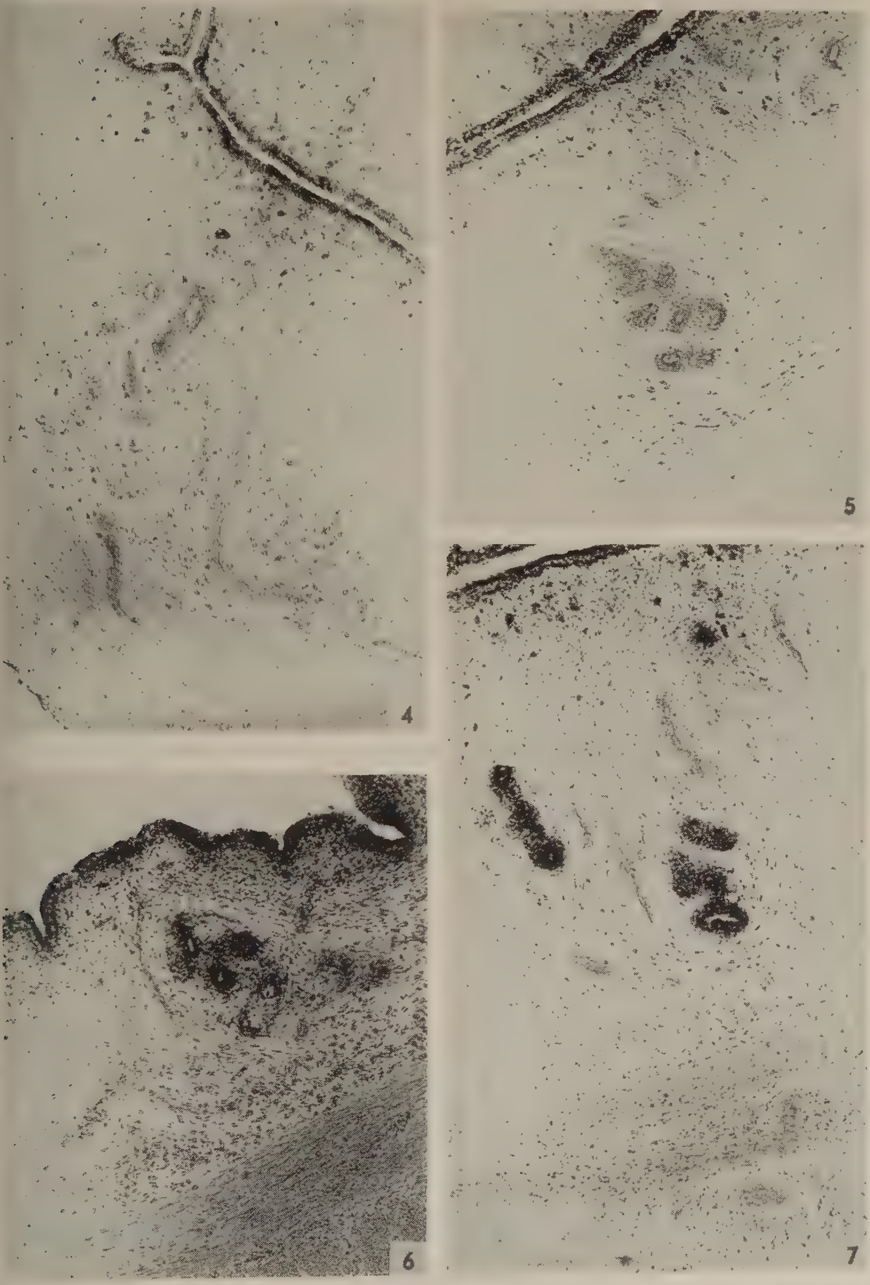
FIGURE 4. Thirty-six-hour diestrus. The DPN activity of the uterus at this stage is less in the luminal and glandular epithelium compared with that of metestrus uteri. In most cases the muscularis also shows a slight decline in activity.

FIGURE 5. Sixty-hour diestrus. A decreased DPN staining in the luminal epithelium and muscularis is evident. The latter is so weak in activity that it can hardly be recorded photographically (lower left).

FIGURE 6. Proestrus. The pattern of diformazan deposition in this "ballooned" uterus as a result of SDH activity is quite similar to that shown in FIGURE 1. However, darkening of the stroma is a result attributable to light density rather than enzymatic activity. Note the unreactive cells in the stroma just above the muscularis. These exogenous cells are believed to be mast cells.

FIGURE 7. Late proestrus. Compare the activity in this uterus with that in FIGURE 6. A decreased staining of the muscularis and luminal epithelium is evident.



Figures 4 to 7

during estrus when portions of luminal epithelium and glands lost some of the SDH activity present during proestrus (FIGURE 8). The most obvious drop in reactivity was apparent in the muscularis. In this tissue, the staining reaction was a very pale, diffuse pink, so weak in intensity that it was impossible to record photographically.

Metestrus was particularly characterized by a resumption of some SDH activity in the muscularis (FIGURE 9), a condition reminiscent of the DPN study. All other components generally retained the activity present during the estrous stage, although a slight decline in diformazan deposition in the luminal epithelium became evident in certain regions.

A drop in SDH activity during 36-hour diestrus (FIGURE 10) manifested itself clearly in the luminal epithelium. More subtle losses in activity were seen in some glands. The intensity of staining decreased in the muscularis.

No significant difference in SDH activity of the luminal epithelium occurred in 60-hour diestrus (FIGURE 11) when compared with that seen 24 hours earlier, but a general decrease in the reactivity of glands and muscle was discernible.

Vagina

DPN-diaphorase. FIGURES 12 to 23 illustrate the deposition of diformazan as a result of DPN activity in the vaginal epithelium during the estrous cycle. At preproestrus (FIGURE 12), intense activity was present in the basal epithelial layers. This activity decreased progressively in the intermediate cell layers in which initial cornification occurred. The luminal layer of mucus-secreting cells was quite active for DPN, although inferior in reactivity to the stratum germinativum. During proestrus (FIGURE 13) the cells of the germinative layer retained their diformazan content while, in the increasing areas of cornification in the intermediate layer, enzyme activity was absent. The mucified cells in the luminal layer either maintained or possibly increased their diformazan deposits. Estrus was characterized by an increased number of cornified cells adjacent to the vaginal lumen that did not reduce NTZ (FIGURE 14). Cells in the intermediate layers were slightly more reactive than the basally located germinative cells. A sharp decline in DPN activity was evident at metestrus. In general, enzymatic activity at this time was fairly uniform throughout the vaginal epithelium (FIGURE 15). No apparent differences in diformazan content and distribution could be detected between this stage and 36-hour diestrus (FIGURE 16). Most of the vaginas studied at 60-hour diestrus (FIGURE 17) tended to show a slightly increased amount of activity over that seen during the previous stage of early diestrus.

Succinic dehydrogenase system. SDH activity in the vagina was generally

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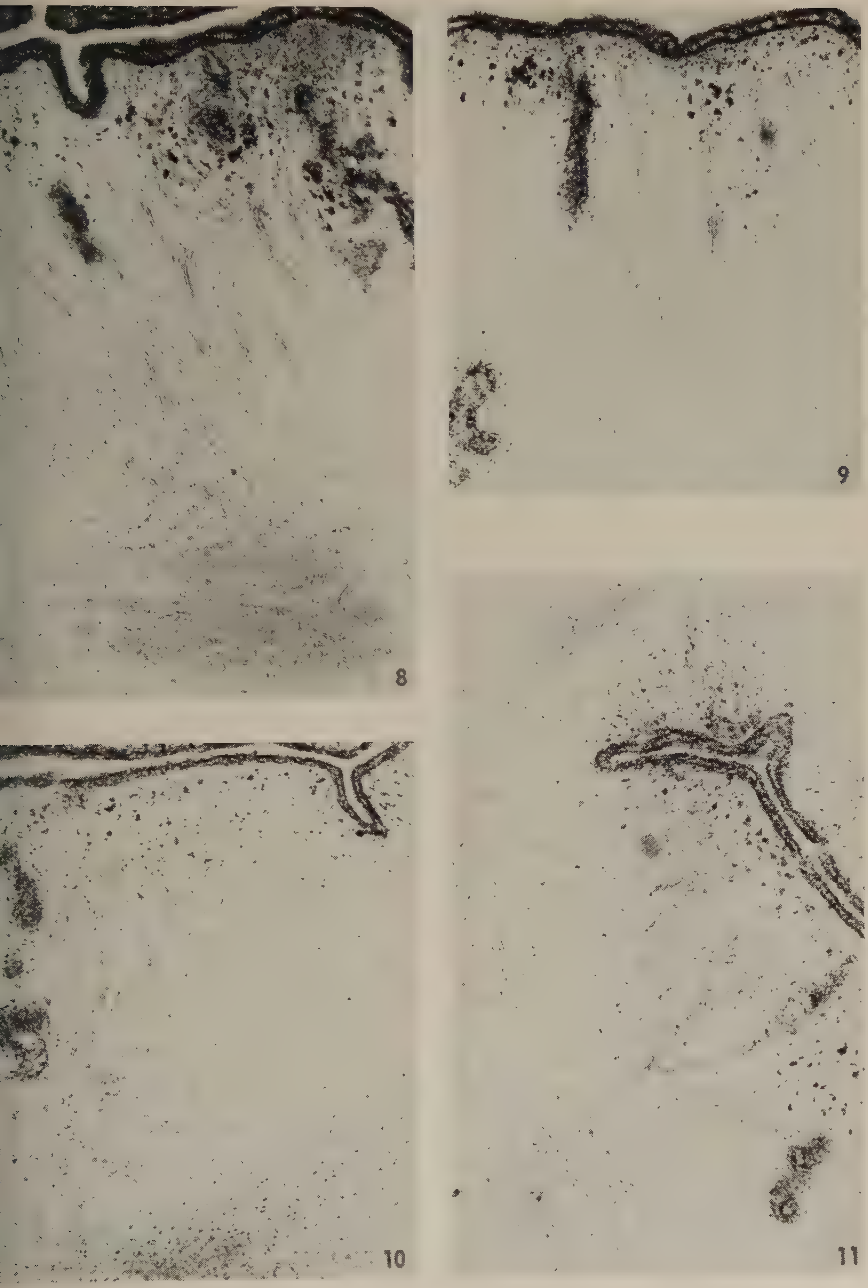
FIGURE 8. Estrus. The SDH activity in the luminal and glandular epithelium is quite high. A drop in activity of the muscularis compared with that in FIGURE 6 also can be seen.

FIGURE 9. Metestrus. SDH visualized here is similar to FIGURE 8, with the exception of a higher degree of NTZ reduction in the muscularis (not seen).

FIGURE 10. Thirty-six-hour diestrus. At this stage, activity is decreased in the epithelium, in some glands, and in the muscularis (bottom of photograph).

FIGURE 11. Sixty-hour diestrus. Luminal epithelium appears unchanged over the former stage (FIGURE 10). Decreased activity is evident in many of the glands and muscle.



Figures 8 to 11

low throughout all the stages of the estrous cycle (FIGURES 18 to 23). The mucus-secreting cells of preproestrus and proestrus, as well as the cornified cells appearing during these stages and estrus, contained no detectable enzymatic activity (FIGURES 18 to 20). A peak reaction in the germinative and intermediate epithelial layers was displayed at proestrus, with an obvious drop at estrus. The germinative cells in the latter stage (FIGURE 20) appeared to be less active for SDH activity. A slight drop in enzymatic activity was observed in metestrous vaginas (FIGURE 21), and this condition appeared to be maintained up to 36-hour diestrus (FIGURE 22). Vaginas from animals in the stage of 60-hour diestrus had a minimal SDH activity, with most of the enzymatic response appearing in the stratum germinativum (FIGURE 23).

Discussion and Conclusions

The study of the transfer of energy in the form of electron potential to that of energy-rich phosphate bonds and of the detailed mechanisms involved in the transfer of such parceled energy into the domain of cell function and maintenance comprises one of the most stimulating and vital areas of investigation in contemporary biochemistry. Enzyme systems such as SDH and DPN represent systems involved in such energy transfer reactions. The histochemical distribution of SDH and DPN should indicate those tissues within a given organ that possess maximal or minimal enzymatic activity. Furthermore, with the application of such techniques to the study of structures such as the normal uterus and vagina, some information should be obtained concerning the individual oxidative metabolism of specific histological entities during cyclic changes in the circulating titers of ovarian steroids.

The normal, cyclical uterus of the rat has been studied biochemically by Bever *et al.* (1954) for succinic and lactic dehydrogenases. For the most part, our own findings agree with the quantitative data on the changing oxidative metabolism of this organ as determined by these investigators. Our localiza-

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FIGURES 12 to 17 demonstrate the localization of DPN activity in the vagina of the rat during the estrous cycle and are oriented with the vaginal lumen toward the top and the stratum germinativum and subepithelial connective tissue at the bottom. All magnifications are × 750.

FIGURE 12. Preproestrus. The highest DPN activity is localized in the luminal and germinative cell layers. Initial cornification is apparent in the intermediate layer, where activity is considerably decreased.

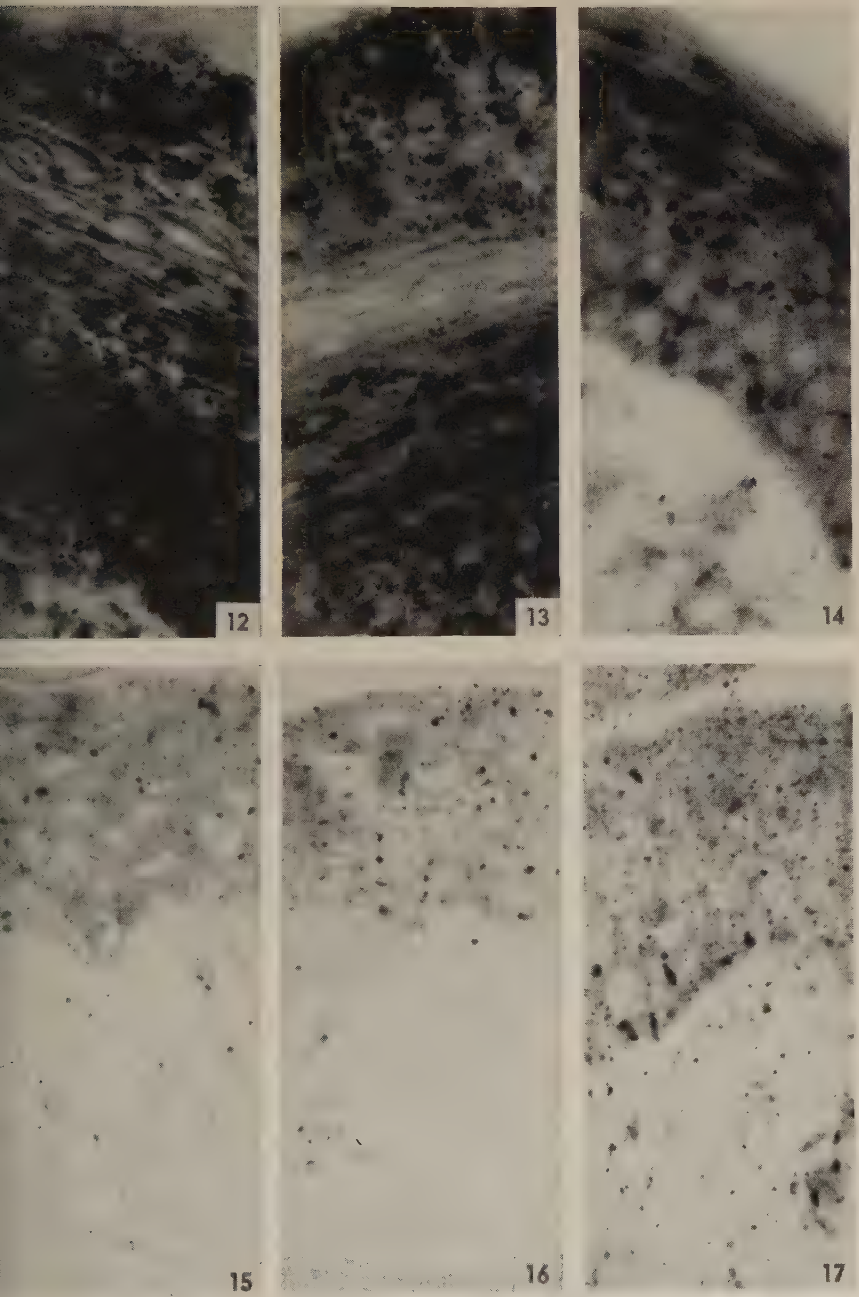
FIGURE 13. Proestrus. The same relative distribution of enzymatic activity prevails here as in preproestrus (FIGURE 12). Note that as cornification increases in the intermediate layer the DPN activity here progressively decreases.

FIGURE 14. Estrus. The highly cornified cells in the upper right-hand corner are completely negative for DPN activity. All activity is localized in the intermediate and germinative layers.

FIGURE 15. Metestrus. Relatively even distribution of enzymatic activity is present. Note the striking drop in diformazan deposition in the regions more active in former stages (FIGURES 12, 13, and 14).

FIGURE 16. Thirty-six-hour diestrus. Hardly any change can be detected between this stage and that shown 24 hours later (FIGURE 17).

FIGURE 17. Sixty-hour diestrus. A generally uniform distribution of DPN activity is apparent, with the possible exception of a somewhat higher deposition of diformazan in the derminative layer. The slightly increased activity of this stage over that of the last (FIGURE 16) can be seen.



FIGURES 12 to 17

tions show that epithelium is the most reactive uterine tissue for DPN and SDH during all phases of the estrous cycle in the rat. This is a reasonable finding and probably can be associated with the fact that demands for synthetic, secretory and, possibly, reabsorptive activity are strong in such regions as the luminal and glandular epithelia. Similar correlations have been indicated by Padykula (1952) in her studies of the distribution of SDH in normal tissues of the rat. The intensity of epithelium staining as a result of SDH or DPN activity during periods of high estrogen secretion (that is, proestrus and estrus) is approximately of the same order, indicating the similarity of enzymatic response during these intervals of the cycle. From this it would be reasonable to assume that homogenate studies of uterine tissue for such enzyme systems reflect, in their quantitative data, oxidative enzymatic activity found chiefly in the epithelial components. With the subsequent decline in activity in these regions of the uterus during later stages of the cycle, one might suspect that the oxidative activity of other areas of the uterus contributes relatively more to the sum total of enzymatic response in this organ.

A differential response of SDH, DPN, and TPN-diaphorase to estradiol stimulation in the uterus and vagina of the mouse also has been reported recently (Rosa, 1957). It would appear, therefore, that the circulating hormonal environment affects various regions of the uterus in a characteristic fashion. Whether these enzymatic responses are related to the specific permeabilities of ovarian steroids by such structures as epithelium, muscle, or stroma remains to be seen. It is important to note, however, that regions showing an absence of staining with these histochemical reactions cannot necessarily be judged as containing no enzyme. The technical manipulations of these procedures sometimes unavoidably involve methods that tend to destroy the enzyme. In all probability, freezing and thawing, a necessary requirement for tissue sectioning, destroy some SDH (Keilin and Hartree, 1949). In spite of this, some factors that could conceivably be operative in effecting tissue differences in SDH and DPN reactiveness might be: (1) real differences in the amount of enzyme, (2) dissimilar pH, substrate, and phosphate concen-

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FIGURES 18 to 23 demonstrate the localization of SDH activity in the vagina of the rat during the estrous cycle. Orientations and magnifications are similar to those in FIGURES 12 to 17.

FIGURE 18. Preproestrus. Luminal and intermediate cell layers are, for the most part, negative. Some activity can be seen in the lower portions of the latter, but the greatest deposits of diformazan prevail in the stratum germinativum.

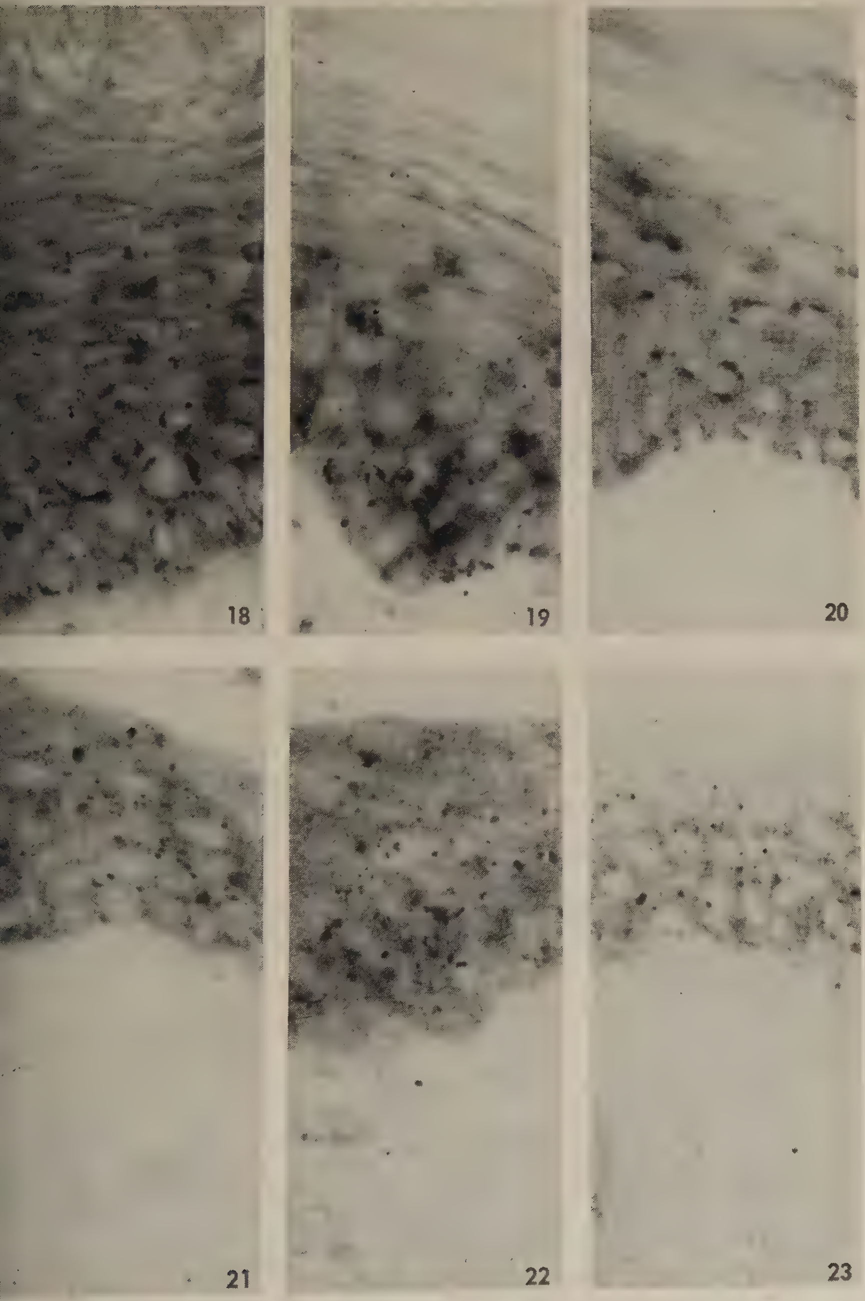
FIGURE 19. Proestrus. Both luminal and intermediate layers are unreactive, with the possible exception of a few of the deeper cells in the latter. Here an increased activity in the germinative layer over that shown in FIGURE 18 can be seen.

FIGURE 20. Estrus. The cornified cells at the top of the photograph are completely negative. The germinative cells are the most reactive, but show a decreased SDH activity in comparison to proestrous vaginae.

FIGURE 21. Metestrus. Activity of SDH is somewhat lowered but evenly distributed throughout all cell layers of the vaginal epithelium.

FIGURE 22. Thirty-six-hour diestrus. Deposition of diformazan as a result of enzymatic activity is very similar to that seen during metestrus.

FIGURE 23. Sixty-hour diestrus. The luminal layer of cells contains less diformazan than the lower cell layers. The decreased staining in this stage of the cycle compared with the earlier stage of diestrus (FIGURE 22) is evident.



Figures 18 to 23

tration optima in individual tissues, and (3) estrogen interactions such as oxidative enzyme inhibition (Villee, 1957a) occurring in some tissues of an organ more readily than in others because of the possible unique character of certain intracellular environments.

An interesting investigation of SDH in the human endometrium has been reported (Foraker *et al.*, 1954), in which no differences in epithelial reactivity could be discerned during the menstrual cycle. In some localizations of SDH and DPN in the uteri of several monkeys (carried out in F. L. Hisaw's laboratory) we have seen indications that degrees in intensity of staining are variable, depending upon the specific hormonal treatment. High estrogen titers in castrates appear to be capable of increasing the enzymatic response in the endometrium of this primate.

The observations recorded in this study also indicate the differential activity of both SDH and DPN in the various tissue components of the vagina during the estrous cycle. As in the uterus, maximal activity for both enzyme systems appears to coincide with periods of the cycle believed to represent high estrogen secretion. Subsequent stages of metestrus and diestrus are characterized by lower enzymatic activity in all regions of the epithelium. It is significant to note the complete refractivity of cornified cells to these dehydrogenase methods; this is probably a reflection of the cessation of metabolic activity in these constituents. Mucified cells show intense reactivity for DPN, while staining, as an indication of SDH activity, is almost if not completely absent. This condition implies strongly the association of DPN activity with mucin production, a feature common not only to the vagina but also to the colon (Rosa, 1954). The similarity between mucin-producing cells of the vagina and the glandular cells of the colon has been stressed by Florey (1932). However, the precise significance of the intense DPN reactivity of these cells and its relation to mucin synthesis is obscure at the present time.

During the long series of reactions leading to the ultimate oxidation of metabolites such as lactate and succinate, energy is formed that ultimately may make its way into the synthesis of high-energy phosphate esters such as adenosine triphosphate (Lipmann, 1946; Sumner and Myrback, 1951; Fruton and Simmonds, 1953). Oxidation-reduction systems such as these have been and still are being studied extensively in order to elucidate the mechanisms by which such available energy is transformed into the physical and chemical work of cells and tissues. In the present investigation an attempt has been made to correlate the hormonal environment of the body with oxidative enzymatic activity of the uterus and vagina. Estrogen-induced growth of the uterus and vaginal epithelium and the stimulation of protein synthesis and mitoses are well known. These responses require the utilization of adenosine triphosphate. The chain of biochemical events that prevails between the estrogenic activation and subsequent responses of target organs such as those considered above are still without precise elucidation. Important advances in our current knowledge of these factors recently have been made by Villee (1957b) and others. These investigators have been able to show in human endometrium and placenta a direct *in vitro* stimulation by estradiol of a cell-free diphosphopyridine nucleotide-requiring isocitric dehydrogenase preparaion (Villee and Gordon, 1956). As these authors point out, this specific action of estradiol would increase the rates of Kreb's cycle activity of other energy-obtaining enzymes. It is quite possible, therefore, that the heightened enzymatic responses we have visualized histochemically are the results of an initial stimulation by estradiol of the isocitric dehydrogenase under study by Villee and his group.

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CYTOCHEMICAL STUDIES ON NONMALIGNANT AND MALIGNANT HUMAN ENDOMETRIA*

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In recent years the human endometrium has been the subject of chemical studies, both cytological and biochemical, with the hope that such studies might have an important bearing on a better understanding of the varied structural and functional changes inherent in this tissue. The endometrium has been of interest from this aspect to some investigators concerned with normal growth and differentiation of tissues, to others because it is a target organ for steroidal hormones, and to clinicians concerned with sterility and other problems of the human female.

The cytochemical studies reported in this paper stem from another field of interest. They represent part of a project designed to correlate histological, cytochemical, and biochemical analyses of gynecological tumors with the different histological grades of malignancy of the tumors. The basic concept of this project has been presented in previous publications.[1, 2]

In brief, the project was planned to correlate cytochemical localization of substances with biochemical determinations in the same tumors. This paper, then, presents some of the current cytochemical studies on endometrial tissues that have been carried out within this larger program. Visual and measurable observations on chemical constituents of endometrial cells, cytochemically defined, will be related to degrees of loss of differentiation in endometrial adenocarcinoma.

The assignment of a histological grade of malignancy, based on degree of loss of differentiation, admittedly has little practical value for many malignant neoplasms. Of recent years, however, most investigators have agreed that grading is a relatively reliable and important factor in determining prognosis for endometrial carcinoma.[3, 4] On the other hand, the difficulty of critical diagnosis in tumors of questionable malignancy, as well as the discriminating cognition of the grade of malignancy, have been acknowledged repeatedly by experienced pathologists and undoubtedly are factors in the divergence of reported cure rates.

These current studies have been carried out under the proposition that assessable differences in cellular constituents between grades of malignancy may be correlated with differences in the same constituents between tissues of benign origin and low-grade tumors. The evaluation of such differences between benign and highly differentiated tissues and among the several grades of malignancy may not only be useful as a better basis for critical diagnosis of malignancy and for prognosis, but also may serve to elucidate fundamental chemical characteristics of malignancy.

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† Damon Runyon Memorial Fund Research Fellow.

MATERIALS AND METHODS

The tissues used in this investigation include seventy-seven cases of non-alignant and forty cases of malignant endometria. These endometria were ken from hysterectomy specimens obtained at operation in the Presbyterian nd Francis Delafield Hospitals, New York, N. Y. Thirty additional control ssues were obtained by endometrial biopsy.

The biopsy material was secured from women with known normal menstrual ycles and was taken on specific days of the cycles. All but 2 of these endo-etria were secretory and ranged from day 16 through day 28 of the cycle. he 77 control endometria procured at hysterectomy were staged for the hase of the menstrual cycle by the criteria of Noyes *et al.*[5] for dating the ndometrial biopsy. In this latter control group, 32 were proliferative, 29 ere secretory, and 16 were postmenopausal endometria. Benign hyperplastic ndometrium was not included in this current series.

Each tumor in the group of adenocarcinomas was assigned a histological rade based on study of hematoxylin and erythrosin sections, according to a lan reported previously.[2] Specific details concerning the grading of endo-etrial carcinoma were included in a recent publication.[6] FIGURE 1 presents hotomicrographs of typical areas, illustrative of the three histological grades f malignancy used as a basis for the cytochemical studies.

Ribonucleic acid (RNA) was visualized in sections and tissue smears by use f the methyl green-pyronin Y method. A detailed account of this method, s used in our laboratory, has been published.[1] In this current study, special mphasis has been placed on the evaluation of differences in the nucleolar RNA mong the types of endometria, nucleoli being identified by the specific reac-on of their RNA content. Biometric analysis of the nucleolar characteristics f each specimen was obtained by measuring the diameters of single nucleoli er nucleus (in 50 to 100 nuclei per case) and by counting the number of mul-ple nucleoli (in 200 nuclei per case).

The carbohydrate, glycogen, and other substances with the 1-2 glycol nkage (glycoprotein) were identified by utilization of the periodic acid-Schiff PAS) method of McManus.[7] For preservation of glycogen, an alcoholic bumen solution was used in spreading the paraffin sections. Serial sections, cceeding those reacted for glycogen, were exposed to enzymatic diastase gestion before application of the PAS solutions. As is well known, this rocedure identifies glycogen in the undigested sections by its absence in the igested sections and establishes the reacted material in the digested sections s glycoprotein. For digestion of glycogen, a 1 per cent solution of malt astase at room temperature, as recommended by Pearse,[8] has been found ore practical than digestion by saliva or by buffered diastase solutions.

The enzyme alkaline phosphatase was demonstrated by Gomori's[9] procedure, nploying refrigerated acetone for fixation, followed subsequently by both dium glycerophosphate and glucose-1-phosphate as substrates. The latter bstrate in paraffin sections produced better delineation of sites of enzyme tivity.

Sites of the succinic dehydrogenase enzyme system were determined by the

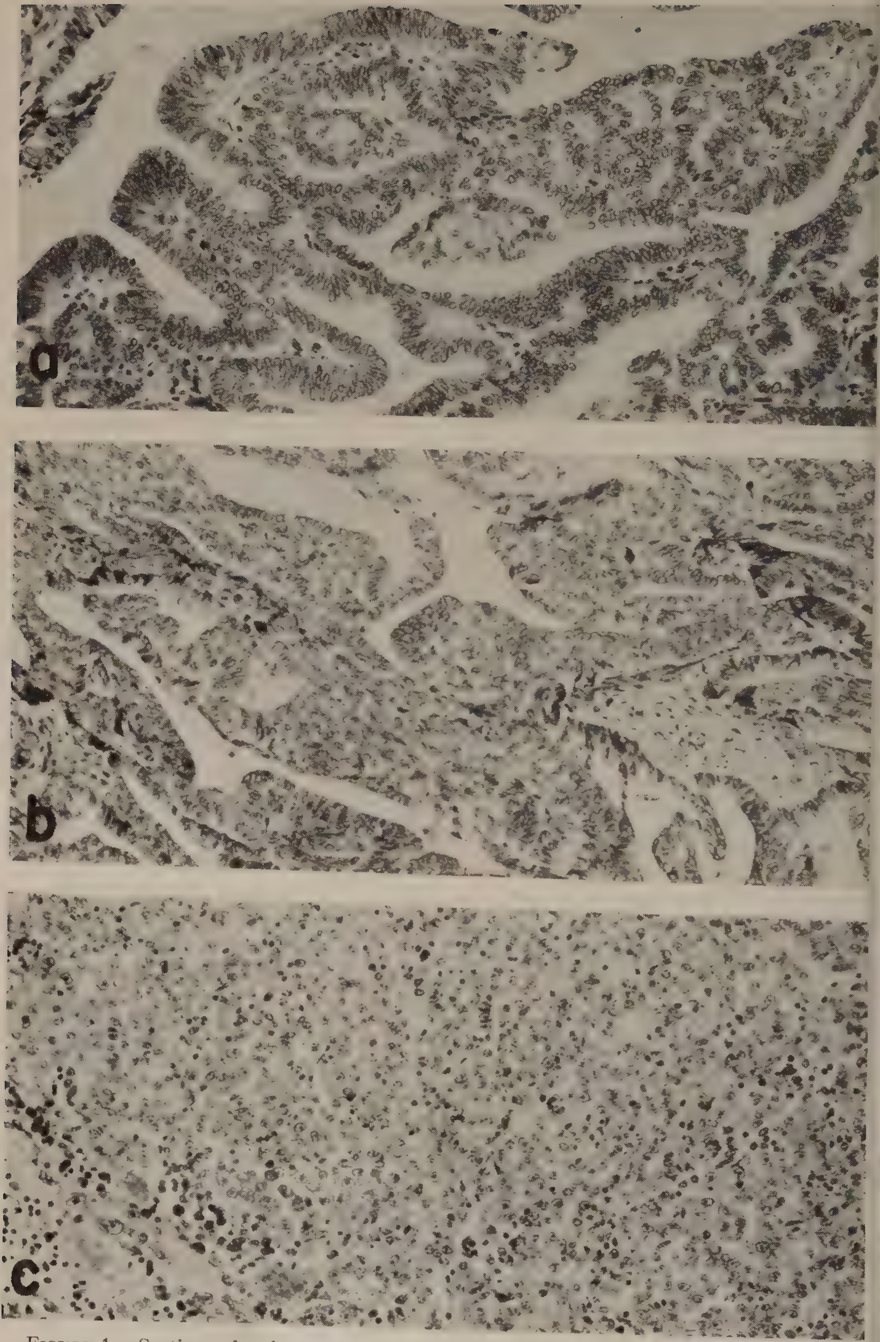

FIGURE 1. Sections of endometrial adenocarcinoma to illustrate histological grades of malignancy. H. & E. ×150. (*a*) Grade I. Glandular elements are presented in one to several layers, with scant stromal tissue between layers. Columnar cell shape is preserved. (*b*) Grade II. Tumor exhibits moderate differentiation, with an over-all appearance of multiple layers. The epithelial cell form tends to be more rounded. (*c*) Grade III. Undifferentiated tumor manifests itself in solid sheets of carcinoma cells, with slight evidence of glandular derivation. Marginal areas of such tumors sometimes show more differentiated regions. Necrotic tissue, characteristically present in undifferentiated tumors, is shown at lower left.

method of Rosa and Velardo,[10] in which neotetrazolium chloride serves as the hydrogen receptor and indicator of dehydrogenase activity. Processing of he human tissues for this enzyme system was initiated as soon as the specimen was removed from the patient and could be taken to the laboratory (within 5 o 15 min.). Such immediate processing was considered essential since *in vitro* biochemical studies on mouse liver have related decreasing succinoxidase activity with length of time after removal of the tissue from the animal.[11]

The friability of human endometrial tissues necessitated the introduction of modifications of the Rosa and Velardo method. Preparation of unfixed, 10-μ frozen sections for incubation was facilitated by placing the tissue in liquid 4 per cent gelatin,[12] with immediate hardening of the block for 15 min. in the refrigerator freezing compartment. An additional aid in the prevention of thawing was the employment of dry ice in a special holder[13] on the knife of the microtome, which is attached to a Techni-Freeze refrigerator unit. This unit recirculates freon gas effectively for the freezing period essential to the destruction of endogenous substrate activity and for cutting the frozen sections.

Lipids were localized in frozen sections of gelatin-embedded tissues using Baker's[14] Sudan black B method, which identifies all liquid lipids. Cholesterol, which occurs as part of the liquid lipid complex in cells, was identified by the Schultz and Lohr histochemical modification of the Lipscheutz's biochemical test for oxycholesterol and by the Schultz modification of the Lieberman test.[15] The blue-green end point color that identifies cholesterol in sections using the above methods cannot be confused with reactions of other unsaturated steroids such as pregnenolone.[16]

Results and Analysis

Ribonucleic Acid

A biometric analysis of nucleolar populations in nonmalignant and malignant endometria is presented in TABLES 1 and 2. A more detailed consideration of this analysis has been furnished in a recent publication.[6]

TABLE 1

ENDOMETRIUM. MULTIPLE NUCLEOLI PER NUCLEUS PERCENTAGE

Histological diagnosis	Number of cases	Number of nucleoli per nucleus				
		2	3	4	5	6+
Nonmalignant	77	78.7	18.4	2.7	0.2	
Proliferative	32	75.9	20.7	3.2	0.2	
Secretory	29	77.9	19.2	2.7	0.2	
Postmenopausal	16	85.7	12.6	1.6	0.1	
Adenocarcinoma	40	25.4	35.6	22.2	8.9	7.9
Grade I	10	22.2	37.1	26.7	9.0	5.0
Grade II	17	28.6	38.9	20.9	7.3	4.3
Grade III	13	23.9	30.0	20.5	10.9	14.7

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TABLE 2

ENDOMETRIUM. SINGLE NUCLEOLI PER NUCLEUS: MEAN DIAMETER

Histological diagnosis	Number of cases	Mean ± SEm (microns)	Grade comparisons	Probability
Nonmalignant	77	1.06 ± .021	Nonmalignant versus Grade I	≪0.001
Proliferative	32	1.11 ± .021	Grade I versus Grade II	0.32
Secretory	29	1.04 ± .018	Grade II versus Grade III	<0.001
Postmenopausal	16	1.01 ± .026	Grade I versus Grade III	<0.001
Adenocarcinoma	40		Nonmalignant versus Grade III	≪0.001
Grade I	10	1.73 ± .094		
Grade II	17	1.85 ± .062		
Grade III	13	2.25 ± .072		

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Counts of multiple nucleoli per nucleus are shown in TABLE 1 as percentages of multiples calculated for all the endometria in the series. In the nonmalignant endometria, 2 to 5 nucleoli per nucleus were observed (FIGURE 2) and only 0.2 per cent of the nuclei contained as many as 5 nucleoli. In the malignant tissues, nucleolar numbers per nucleus ranged from 2 to 16 and more (FIGURE 3) with 16.8 per cent of the nuclei having 5 and more nucleolar bodies.

FIGURE 3e illustrates a single cell from an ascites fluid preparation obtained from a case with recurrent Grade III endometrial carcinoma. It cannot be definitely ascertained whether this was a multinucleate cell or whether the lines indicative of separate nuclei were folds in the nuclear membrane. At any rate, the nucleolar population of this interphasic cell was composed of more than twenty-five pyroninophilic nucleolar structures. The full complement of numerous nucleoli per nucleus cannot be illustrated adequately, since the nucleolar units lie in different planes of focus.

TABLE 1 not only shows an incidence of more nucleoli per nucleus in malignant endometria than in nonmalignant tissues, but also demonstrates a trend of higher nucleolar multiplicity with increased grade of malignancy.

It is shown in TABLE 2 that single nucleoli per nucleus were smaller in the nonmalignant than in the malignant endometria, and that the size of single nucleoli increased with increased grade of malignancy (compare FIGURES 2 and 3). Probability comparisons between groups reveal that, with respect to mean single nucleolar diameters, there was a statistically significant difference between group nucleolar populations for all groups except Grades I and II. The chance occurrence of the difference between the means was most remote between the proliferative and Grade I adenocarcinoma groups also.

Included in the Grade I group by histological criteria were two tumors with nucleolar characteristics of Grade III. This situation undoubtedly accounts for the larger SEm of the Grade I group (TABLE 2), for the lack of significance between Grades I and II, and for the occurrence of a higher percentage of multiple nucleoli in Grade I than in Grade II (TABLE 1). It may be that the

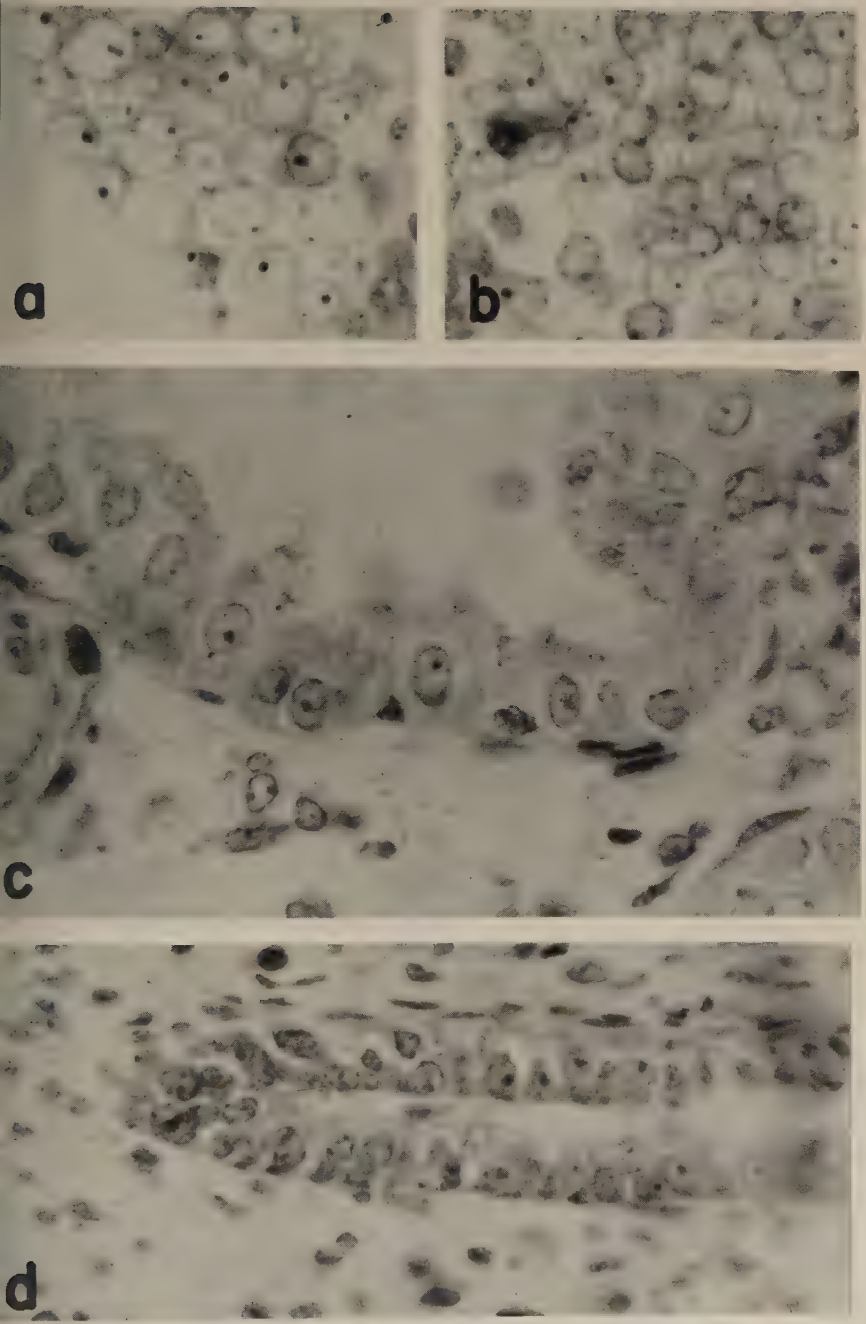

FIGURE 2. Nonmalignant endometria. Tissue smears are advantageous for nucleolar udies, since they present whole nuclei for nucleolar analysis. Methyl green-pyronin Y.) Proliferative tissue smear; (*b*) secretory tissue smear; (*c*) secretory section; (*d*) atrophic ection, postmenopausal. Compare the smaller sizes and more regular form of single nu leoli in this figure with those in FIGURE 3. Note the small multiples, 2 to 4, in *a*. ×918.

nucleolar index provides a more reliable measure of degree of malignancy than the more subjective histological criteria.

In Grade III tumors it was common to observe nuclei with the nucleolar characteristics of Grade I (FIGURE 3). The more differentiated tumors, however, rarely showed nuclei with the nucleolar features of large size and multiplicity representative of Grade III.

Confirmatory evidence that nucleoli of endometrial carcinoma cells were larger than their normal and benign homologues was reported by earlier investigators[17, 18] who established the fact that nucleolar-nuclear ratios were smaller in endometrial carcinoma than in normal regenerating endometrial tissues.

Accompanying larger size of single nucleoli and more multiple nucleoli with increased loss of differentiation, endometrial tumors exhibited more variations in size and in regularity of form in nucleoli. In contrast, the nucleolar populations of tissues of origin presented fewer and smaller nucleoli that were almost always rounded or oval in shape. Although fixed preparations form the basis of these findings, similar observations on living malignant cells by investigators[19, 20] in other fields permit the implication that this reported enhancement of nucleolar alterations with loss of differentiation indicated a corresponding increase in nucleolar activity.

Associated with greater nucleolar activity as reflected by these morphologic observations were increased amounts of cytoplasmic RNA. Glandular cytoplasmic RNA was observed to be more abundant in proliferative endometrium, in which the mean nucleolar diameter was 1.11 μ (TABLE 2), than in secretory and atrophic endometria, in which the mean diameters were 1.04 and 1.01 μ, respectively. The biochemical data obtained by Stein and Stuermer,[21] who reported a peak of ribonucleoprotein in the late proliferative phase, with decreasing amounts as the secretory phase progresses, may be assumed to reflect the current cytochemical demonstration of larger nucleoli and more cytoplasmic RNA in the first half of the menstrual cycle.

Subjective estimations of cytoplasmic RNA in the adenocarcinoma groups indicate an over-all increase over that observed in the nonmalignant control series. It is considered, however, that subjective estimations, inherent in cytochemical observations, are not sufficiently discriminating to estimate differences among grades of malignancy. Other investigators[22-25] have reported similar findings for cytoplasmic RNA during the menstrual phases and in carcinoma.[22, 23, 26] McKay *et al.*,[26] however, did not find the RNA substance consistently present in adenocarcinoma.

Since the nucleolus is known to be the site of RNA in the interphasic nucleus[27, 28] and is considered to be the major RNA-producing organoid of the cell,[19] the observations included in this report may imply an increase in RNA between benign and low-grade malignant endometrial tissues, as well as between the several grades of malignancy among the tumors studied. Similar nucleolar findings have been reported by our laboratory for ovarian papillary serous tumors.[1]

This type of cytochemical investigation yields only suggestive evidence that larger, more numerous, and irregular nucleoli, with their inference of nucleolar ctivity, are indicative of augmented production of cytoplasm RNA and of inrease in RNA with dedifferentiation. Correlative biochemical work within he larger program of this project[29] and quantitative data on animal and other uman tumors[30] support this implication.

Glycogen and Glycoprotein

The distribution of glycogen and glycoprotein in nonmalignant endometrium s too well known to merit further consideration here. In agreement with the bservations of others,[22, 25] glycogen occurred in scant subnuclear traces in the epithelium of the proliferative phase, with more glands showing minimal basal droplets as the phase progressed. The stromal cells were negative for glycogen throughout the proliferative stage. Glycoprotein was minimally present in some cases at the apical tip of gland cells and in the gland lumens.

In early secretory endometrium (FIGURES 4a and 4b) glycogen appeared as the well-known basal deposits in the epithelial cells, and as fine droplets in the stromal cells. The presence of glycogen in stroma cells was characteristic of all secretory endometrium. As the secretory phase advanced, glycogen was observed in increasing amounts, apically in the glandular cells, in the luminal secretion, and in predecidua cells, as well as other stroma cells. Glycoprotein also was detected in increasing amounts during the development of the secretory phase, and became most prominent in the luminal secretions.

Biochemical data[31] on the glycogen content of normal endometria demonstrate a gradual increase in glycogen during the proliferative phase, a pronounced rise at day 17, a drop in value to day 21, followed by a slight premenstrual rise. Subjective assessment of amounts of glycogen in PAS-reacted sections presents a glycogen peak somewhat later in the cycle.

In the endometrial carcinomas, glycogen presented the irregular distribution which has been reported by others.[26, 32] It was absent in some tumors and abundantly present in others, was observed in both scant and large amounts in different areas of the same tumor, and was either absent or barely present in the stroma cells. Glycoprotein in the tumors presented a similar variable distribution. No correlation existed between amounts of glycogen and glycoprotein and loss of differentiation in the tumors.

Alkaline Phosphatase

The reader is referred to other publications for consideration of the distribution of alkaline phosphatase in nonmalignant endometria.[22, 25, 33] The current observations on activity of this enzyme in endometrial carcinoma also are in agreement, on the whole, with the pattern of localization described by others.[22, 26, 34]

In the malignant tumors the activity of the enzyme was confined to the luminal borders of the epithelial cells, with scattered evidence of its presence in the secretions of the more differentiated tumors. In Grade I tumors in which the glandular character of the neoplasm was most evident, it was irregularly but rather constantly present apically in the cells lining the glands. In Grade II tumors in which multiple layers of cells occurred, it was more variably present at the free borders, and it sometimes occurred within the layers when cells lined small luminal spaces. In Grade III tumors it was not present in solid sheets of carcinoma cells, but was present inconstantly at the apical ends of cells that bordered spaces. In general, then, alkaline phosphatase activity appeared to decrease with increased grade of malignancy in endometrial tumors. It was present, however, irregularly, in all grades.

Succinic Dehydrogenase System

In our hands, the use of the Rosa and Velardo method results in preparations in which diformazan deposits appear in the form of single separate granules and rods and of multiples of these structures in the cytoplasm of cells. Under low powers of the microscope the deposits have the appearance of clumps of the substance. Nuclei do not show evidence of activity of this enzyme system. The reaction occurs predominantly in the epithelial elements in human endometrial tissues, with a less intense reaction in the stroma cells.

The glandular cytoplasm of proliferative and secretory endometria showed multiple sites of deposits (FIGURES 5a, 5b, and 5c; FIGURE 6a); atrophic endometrium, scanty sites per cell (FIGURE 6b); and endometrial carcinomas of all grades, both types of deposits (FIGURES 6c and 6d; FIGURES 7b and 7c). In FIGURE 5 the reaction in the stroma appears rather uniformly distributed and does not seem to be occasioned by diffusion of the enzyme system. The postmenopausal endometrium from a patient with an ovarian thecoma showed a proliferative type of diformazan deposit similar to that shown in FIGURE 6a. FIGURE 7c illustrates a Grade I carcinoma with a necrotic area at the right. The stroma in the center of the illustration had been somewhat distorted in cutting. In the necrotic area and in similar areas of other tumors, activity of the system was absent. Similar observations have been reported for human heart musculature[35] and have been noted for our series of ovarian tumors.

Biochemical data by Stuermer and Stein[36] have demonstrated an increase in succinic dehydrogenase activity during the proliferative phase, a marked increase during the early secretory stage, with a drop in activity thereafter. Foraker *et al.*,[37] from their histochemical study of normal and hyperplastic endometrium, observed no general over-all variation in activity with the stage of the menstrual cycle. Observations made in this current work are in agreement with this statement.

We believe that subjective estimation of amounts of succinic dehydrogenase activity cannot be made with any degree of accuracy on reacted microscopic sections. Increased deposits of diformazan do not necessarily mean heightened activity of this enzyme concerned with cellular metabolism. Observations, however, on amounts of formazan deposits produced by reaction with neotetrazolium chloride have led to the following conclusion, provisionally, for the current work. Since the cells of all endometrial tissues demonstrated similar amounts and types of formazan deposits, within the limits of subjective estimation of succinic dehydrogenase system activity, these studies have produced no evidence of differential correlation of such activity with grades of malignancy.

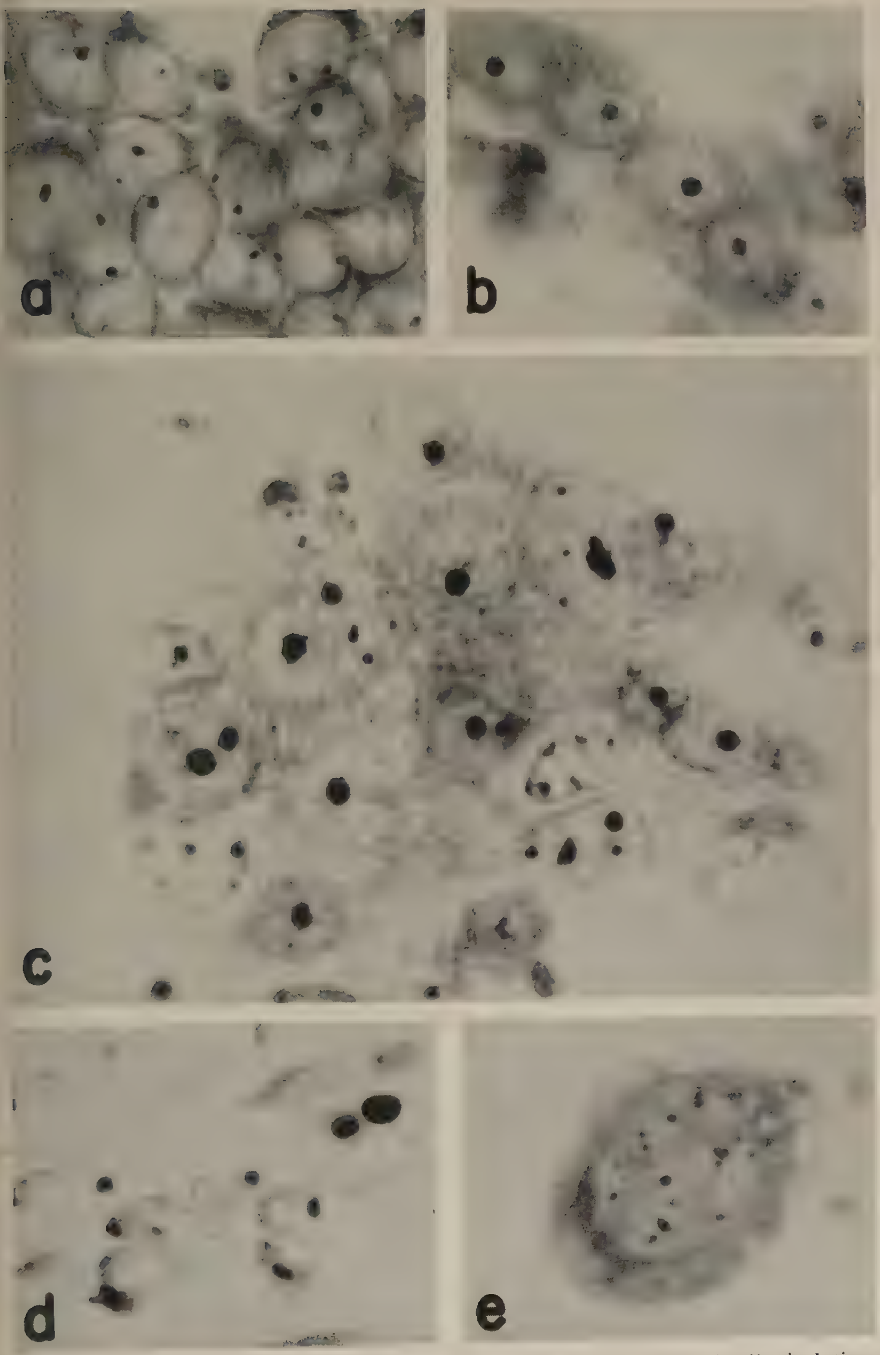

FIGURE 3. Endometrial adenocarcinoma. Methyl green-pyronin Y. (*a*) Grade I tissue smear; (*b*) Grade II tissue smear; (*c*) Grade III tissue smear; (*d*) Grade III section; (*e*) Grade III ascites fluid cells, described in the text. Note the increased size of single nucleoli with increased grade of malignancy, and the multiplicity of nucleoli in Grade III, together with morphologic variations. ×918.

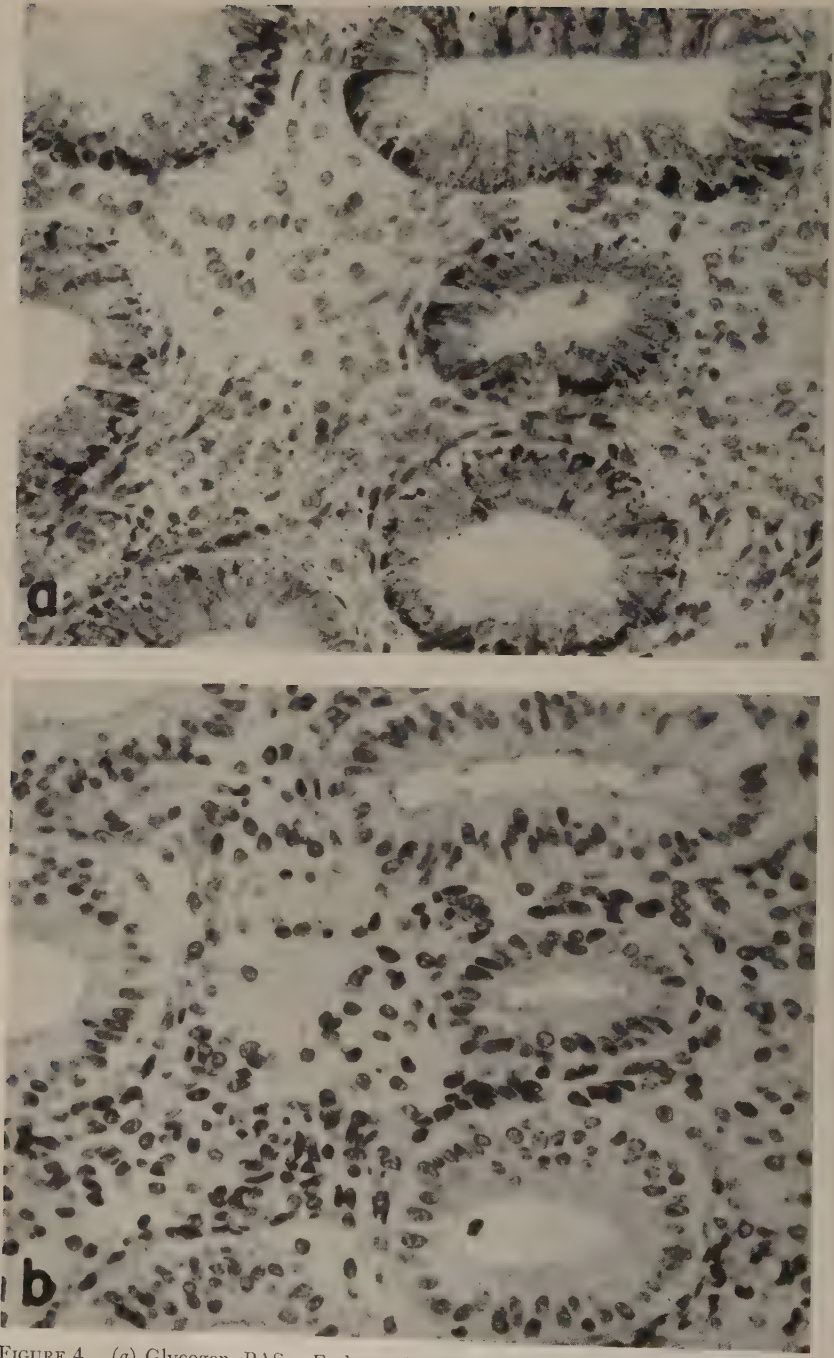

FIGURE 4. (*a*) Glycogen, PAS. Endometrial biopsy, day 16 of normal cycle. Glycogen appears mainly in a subnuclear position in the epithelium. Some glandular cells show its extension through the cytoplasm toward the apical end of the cell. Note the presence in the stromal cells of fine deposits of glycogen. (*b*) PAS, control, diastase digested. Note the absence of glycogen and the scant presence of glycoprotein at the luminal edge of the epithelial cells. ×396

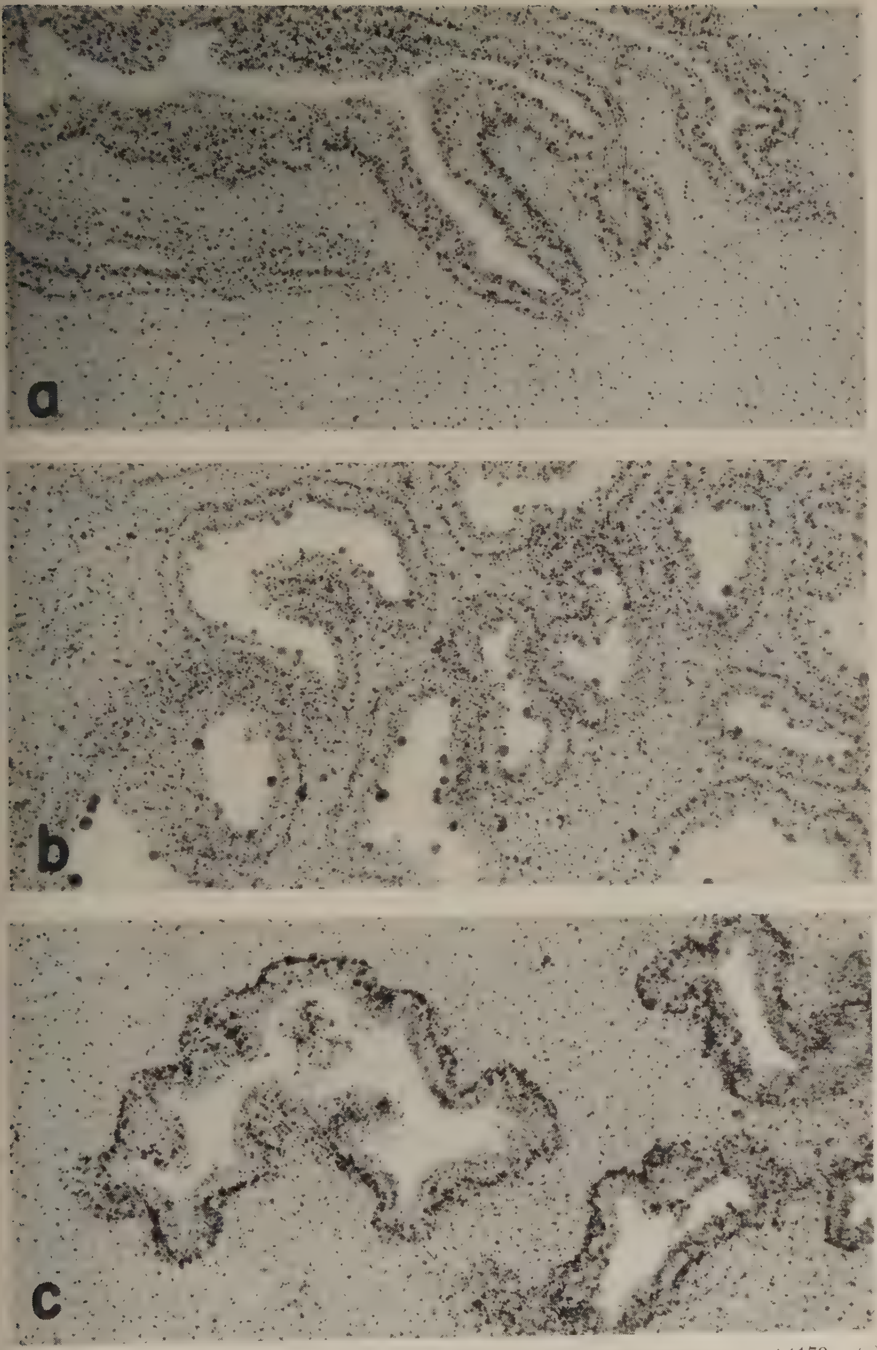

FIGURE 5. Nonmalignant endometrium. Succinic dehydrogenase system. ×170. (*a*) Late proliferative; (*b*) early secretory; (*c*) late secretory. These low-power views illustrate the topographical distribution of enzyme activity in normal endometrium. The luminal structures appearing in *b* represent pink monoformazan droplets. All illustrations in FIGURES 5, 6, and 7, excepting FIGURE 6*c*, are of 10-μ frozen sections not fixed before cutting.

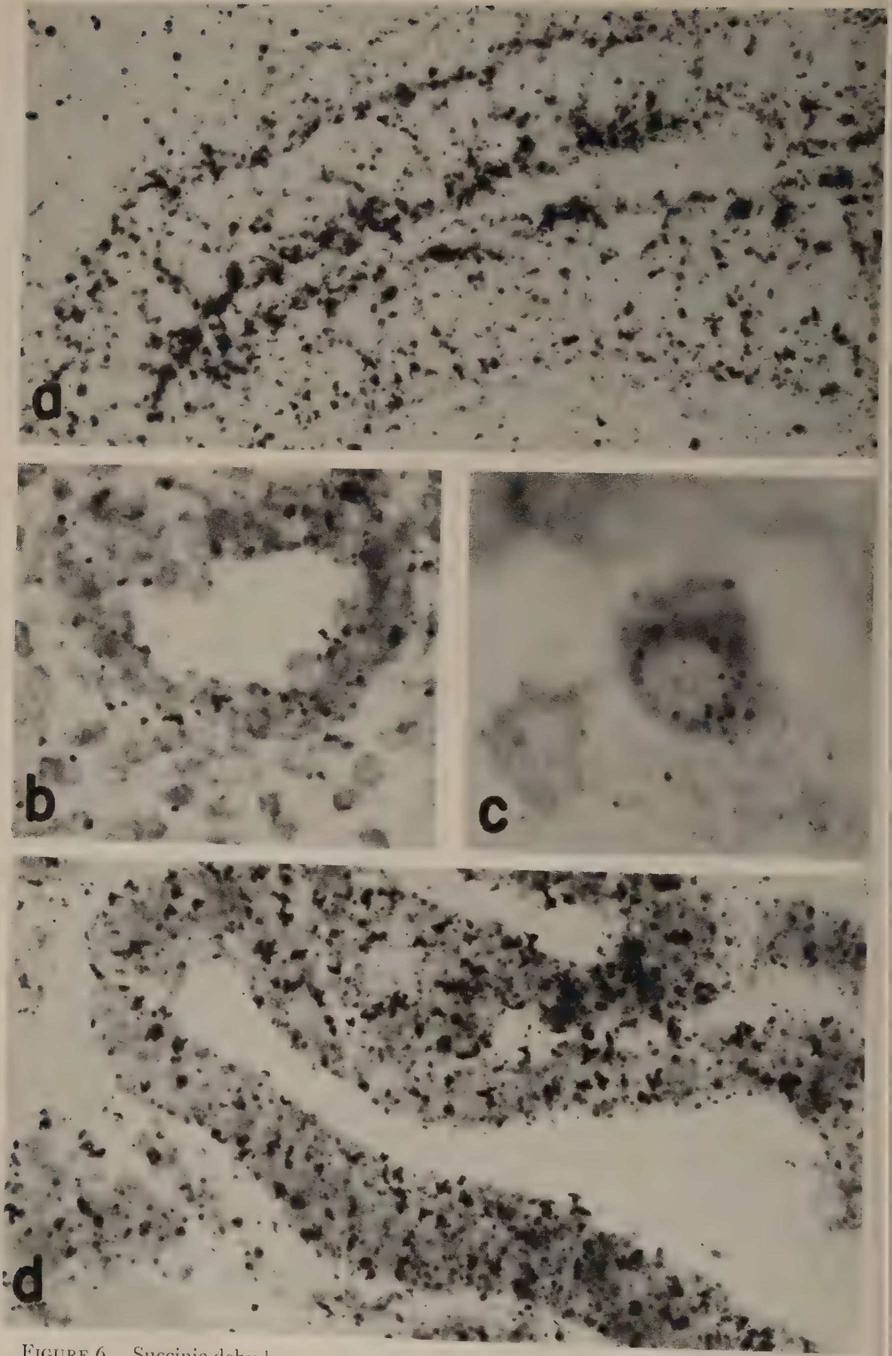

FIGURE 6. Succinic dehydrogenase system. (*a*) Higher power of FIGURE 5*a*. Note single and multiple diformazan deposits predominantly in cytoplasm of glandular cells. Clear spaces represent unstained nuclei with no reaction. ×684. (*b*) Atrophic postmenopausal gland, showing scant deposits of single and multiple structures. Less reaction in cytoplasm of stroma cells than during phases of cycle. ×684. (*c*) Cells from a Grade III tumor tissue smear. ×918. Deposits approximate mitochondria in the cell on the right. The necrotic cell on the left shows absence of activity. (*d*) Grade I tumor, demonstrating single and multiple deposits similar in distribution to that in the proliferative phase. ×684.

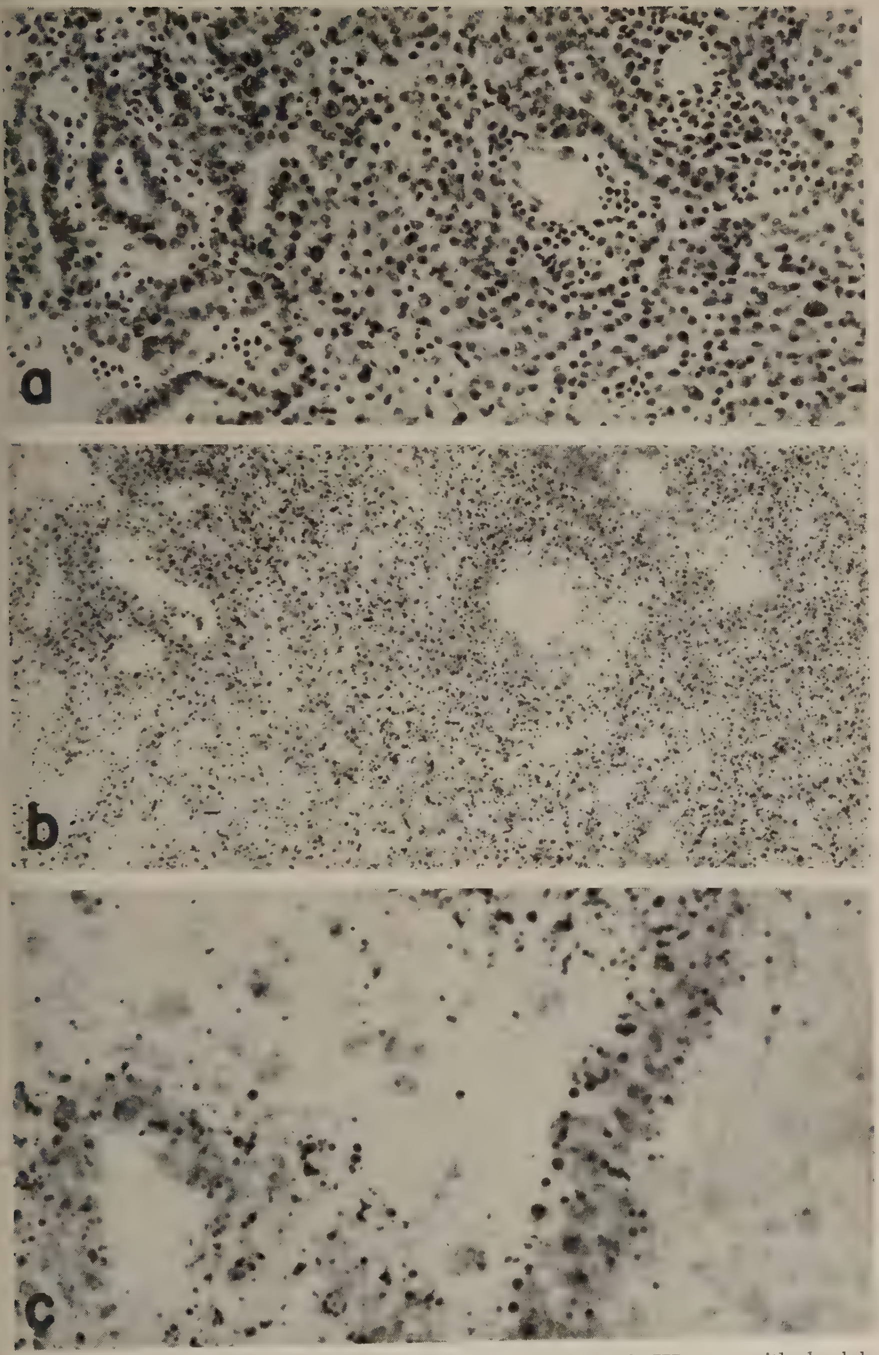

FIGURE 7. FIGURES 7*a* and 7*b* are successive sections of a Grade III tumor with glandular origin evident. ×170. (*a*) H. & E.; (*b*) succinic dehydrogenase system. Low-power view to show the topographical distribution of activity in comparison with stained section in *a*. Note the negative reaction in the vessel and gland lumina and the clearer areas representing thin strands of stroma. There is approximately a similar reaction per cell as in other viable endometrial tissues. (*c*) Succinic dehydrogenase system in a Grade I tumor, demonstrating a necrotic area on the right with absence of enzyme activity. ×684.

Lipids

In normal endometria, sudanophilic lipid may be found in the stroma and in the glandular cells, where it may be basal or apical in location, or throughout the cytoplasm. A number of the specimens in the current series, obtained from women with histories of normal menstrual cycles, and dated for varying days of the cycle, were devoid of lipid.

No glandular lipid was observed in proliferative endometria; scant stromal lipid was detected occasionally in this stage.

In secretory endometria, epithelial lipid, when present, tended to be basal in position (FIGURE 8a). It was never present in all glands of the section. Basal fat was observed in a day-16 endometrium, but was absent in 12 specimens dated from day 21 through day 26 of the cycle, during a period in which Gilman[38] stated there was a remarkable accumulation of basal fat. Stromal lipid was present in 9 of these cases, so that failure of technique could not be incriminated for the absence of epithelial lipid.

The subsurface stroma cells of sections of day-16 endometria contained numerous fine lipid droplets; farther from the surface in these sections fewer droplets per cell were seen. Early predecidual cells had a somewhat increased lipid content, which was decreased (FIGURE 8b) or absent toward the end of the cycle. More stromal lipid sometimes was observed in the functionalis (FIGURE 8c) of mid-secretory endometrium than was noted in sections of early secretory tissue.

Sudanophilic lipid, then, is scant or absent during the proliferative phase of the cycle and is variably abundant during the secretory phase. These observations agree in general with the cytochemical findings of other investigators[22, 38] and with the study of McLennan and Koets,[39] who found that lipids exhibited a chemical pattern throughout the menstrual cycle similar to the cytochemical distribution described above.

Lipid was demonstrated in tumors of all three grades of malignancy. The Sudan reaction was strong consistently in stroma cells and present in some of the glandular elements (FIGURES 9a and 10a), an observation previously recorded by Atkinson.[22]

A few sections of secretory endometria were subjected to the test for cholesterol. Some of the subsurface stromal liquid lipid droplets presented the blue-green color characteristic of the cholesterol reaction. It is recognized that when this sterol or its esters are present in minimal quantities in tissues, the cytochemical detection is not always possible.[16] This sterol was found to be a prominent component of the sudanophilic lipid complex in the stroma of endometrial adenocarcinoma (compare FIGURES 9a and 9b). Sections of Grades I and II (FIGURES 9b and 9c) demonstrated the presence of more cholesterol than sections of Grade III, in which stroma is minimal. FIGURE 10 presents the sudanophilic lipid and the cholesterol reactions in sections of the same area of a Grade-I tumor. It is evident that not all Sudan-reacted droplets in stroma and epithelium presented the cholesterol reaction. It appears from these preliminary cytochemical observations that the stroma of malignant endo-

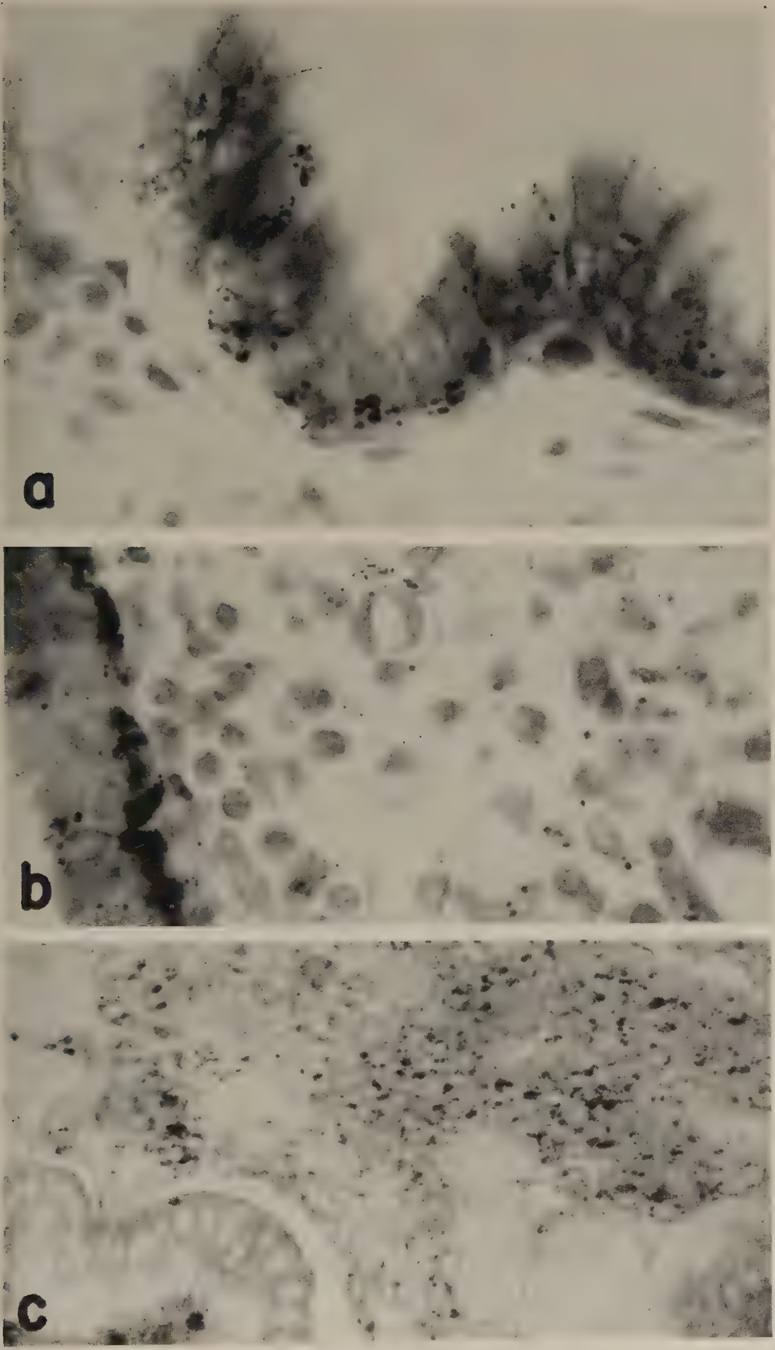

FIGURE 8. Normal endometrium. Lipids. Sudan black B-Hemalum. (*a*) Secretory, days 26–27. Glandular lipid is mostly basal in position. Some apical lipid is evident. Glandular lipid is not present in all glands of this endometrium. There is no stromal lipid in this area. ×594. (*b*) Surface epithelium of the same endometrium, showing scant lipid in the predecidua cells. Note the prominent basal lipid droplets in the luminal epithelium. ×594. (*c*) Secretory, day 20–21. No hemalum nuclear stain. There is considerable stromal lipid present in this endometrium. No glandular lipid was observed in this section. ×396.

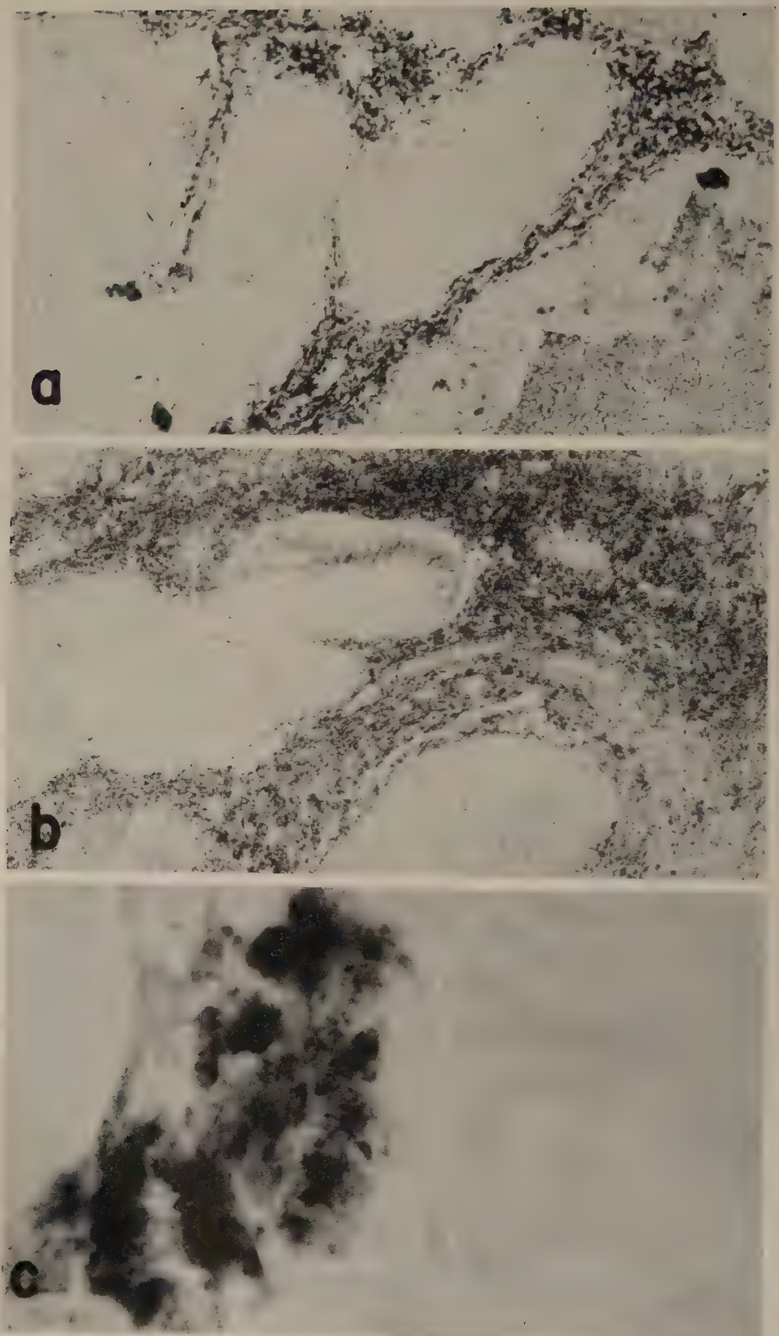

FIGURE 9. FIGURES 9*a* and 9*b* show lipids in Grade I endometrial carcinoma. (*a*) Sudan black B lipid is prominent in the stroma. No glandular lipid was demonstrated in this case. ×125. (*b*) Same case as in *a*. Cholesterol is prominent in the stroma. During reaction, the same lipid droplets that test positive for general lipids with Sudan black B present the blue-green color characteristic of cholesterol. ×125. (*c*) Cholesterol in the stroma of a Grade II tumor Unreacted glandular epithelium to the right and left of the stroma. ×800.

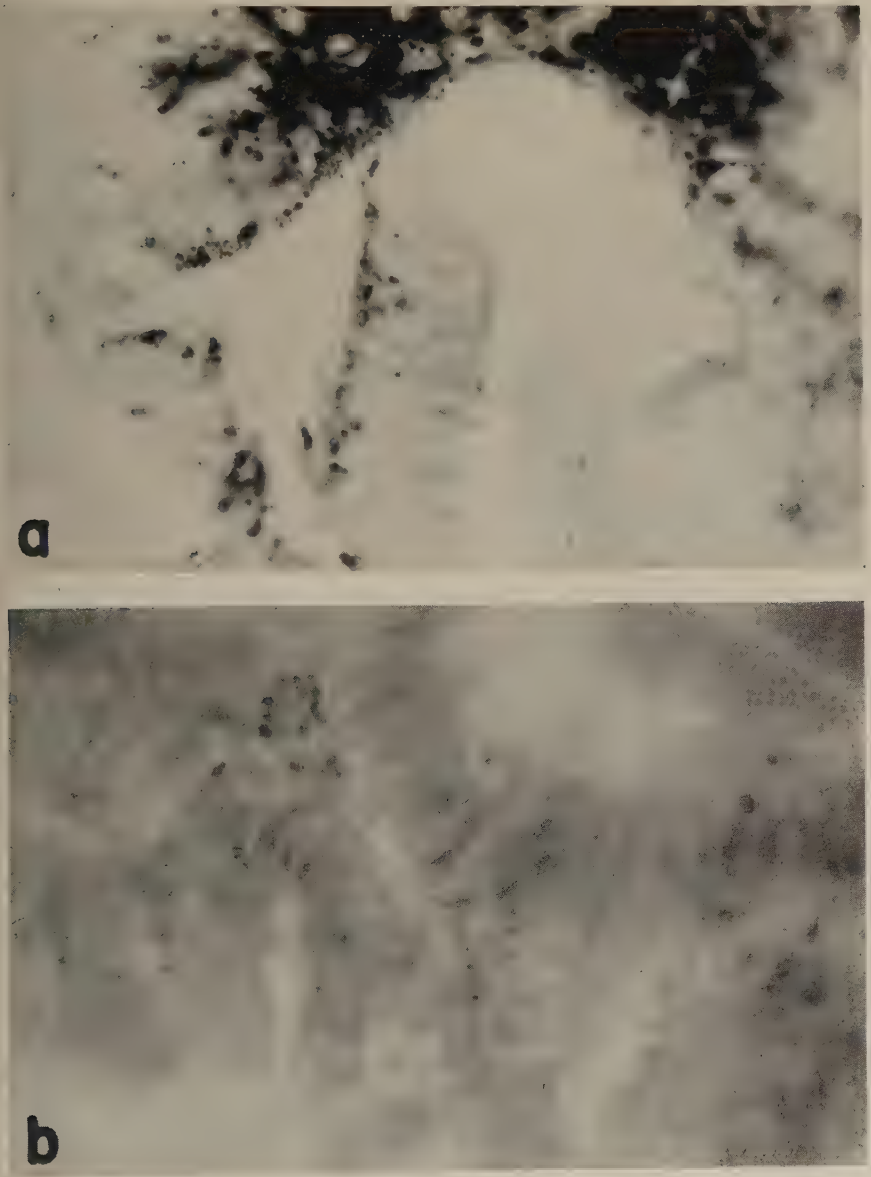

FIGURE 10. Lipids in a Grade I endometrial carcinoma. ×396. (*a*) Sudan black B liquid lipid reaction is present both in the connective-tissue stroma and in the cytoplasm of some of the glandular cells. (*b*) Cholesterol reaction in the same area is positive in some of the liquid lipid droplets of both the stroma and the glandular epithelium.

metria contained more cholesterol than the stroma of nonmalignant endometrial tissues.

Haven and Bloor,[40] in an interesting biochemical review of lipids in cancer, indicated that malignant tumors had much higher contents of cholesterol than did benign tumors, or the tissues in which the tumors grew. This was reported for animal tumors as well as for some human malignant tumors, but did not include endometrial carcinoma.

SUMMARY

This paper has presented cytochemical observations on nonmalignant and malignant human endometria. These studies have attempted to localize specific cellular components of malignant cells and to compare visual or measurable changes in the same constituents in benign tissues of origin and tumors of the several histological grades of malignancy.

Analysis of nucleolar populations in endometrial tissues demonstrated, with loss of differentiation, greater multiplicity, more morphologic variations, and larger size. These nucleolar alterations imply an increase in nucleolar activity and in ribonucleic acid with increased grade of malignancy. Mean diameter of single nucleoli per nucleus showed statistically significant differences, not only between nonmalignant control tissues and low-grade tumors, but also between most of the grades of malignancy. Such nucleolar observations may be of clinical value as aids in differential diagnosis, in selection of therapy, and in serving as a better basis for prognosis.

Other substances—succinic dehydrogenase, alkaline phosphatase, glycogen, glycoprotein—have not been observed consistently to be augmented or decreased with loss of differentiation. Total sudanophilic lipid and cholesterol occurred in increased amounts in the stroma of endometrial carcinoma.

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ESTROGENS AND UTERINE ENZYMES*

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In our original experiments on the mode of action of estrogens, slices of human endometrium were used. It was found that small amounts of estradiol added *in vitro* increase the rate at which oxygen and pyruvic acid are utilized by endometrium.[1] Human endometrium taken at different stages of the menstrual cycle showed cyclic variations in the rates of utilization of oxygen and pyruvate when no estrogen was added *in vitro* which paralleled the amount of estrogen in the blood and urine.[2]

In our subsequent experiments we have used placenta because of its greater availability, but the estrogen-sensitive system appears to be identical in the two tissues.[3-6] Both endometrium and placenta contain an enzyme that is stimulated by minute quantities of estrogen added *in vitro*. Other tissues that appear to contain a similar enzyme are those of the mammary gland, the ventral prostate gland, and the pituitary. In contrast, liver and kidney do not appear to contain this estrogen-sensitive enzyme system.[4]

Our previous experiments on the mechanism of action of estradiol had led to the hypothesis that the soluble protein fraction obtained by high-speed centrifugation of homogenates of human placenta contains a diphosphopyridine nucleotide (DPN)-linked isocitric dehydrogenase that is activated by minute amounts of estrogen added *in vitro*. This same fraction, when incubated with triphosphopyridine nucleotide (TPN) instead of DPN, showed marked TPN-linked isocitric dehydrogenase activity, but the rate of this reaction was unaffected by the addition of estradiol.

In the course of attempts to purify the estrogen-sensitive enzyme of placenta, it was found that the protein precipitated by 0 to 30 per cent saturated ammonium sulfate had no activity alone. It would not oxidize isocitrate when DPN was present in either the presence or the absence of estradiol. The protein precipitated between 30 to 60 per cent saturated ammonium sulfate contained what appeared to be a DPN-linked isocitric dehydrogenase which showed only a slight response to added estrogen. However, when these two fractions were mixed together and isocitrate was added as the substrate, α-ketoglutaric acid was produced in the presence of estradiol, and somewhat less α-ketoglutarate was produced when no estradiol was added. When adenosine triphosphate (ATP) was added to the system the magnitude of the estrogen effect was greatly increased. Adenosine diphosphate (ADP) was recovered at the end of the incubation period and was identified by paper chromatography.

We had interpreted these findings as consistent with the theory that the 0 to 30 per cent ammonium sulfate fraction contains an "activating enzyme" and that the 30 to 60 per cent fraction contains the isocitric dehydrogenase. The

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hormone, we believed, stimulated the activating enzyme to convert an inactive form of the isocitric dehydrogenase to an active form. This was analogous to the theory postulated by Sutherland[7] for the activation of phosphorylase by epinephrine.

In a personal communication, Paul Talalay informed us that he had confirmed our observation that estrogen stimulates the isocitric dehydrogenase system of the placenta. He noted, however, that catalytic amounts of TPN are needed to restore the activity of placental preparations that do not respond to estrogens. A stimulation of the dehydrogenation of glucose-6-phosphate by enzymes from human placenta was also demonstrable in the presence of substrate amounts of DPN and catalytic amounts of TPN. Since estrogen will stimulate glucose-6-phosphate as well as isocitrate dehydrogenation, and since both of these reactions require TPN, Talalay suggested that estradiol stimulates a transhydrogenation between diphosphopyridine and triphosphopyridine nucleotides:

$$\mathrm{TPNH} + \mathrm{DPN}^+ + \mathrm{H}^+ \rightleftharpoons \mathrm{DPNH} + \mathrm{TPN}^+ + \mathrm{H}^+$$

He also suggested that the estrogen stimulation of the transhydrogenation involves a reversible oxidation-reduction of estradiol and estrone:

$$\begin{array}{rl} & \text{Estradiol} + \mathrm{DPN}^+ \rightleftharpoons \mathrm{DPNH} + \mathrm{H}^+ + \text{Estrone} \\ & \underline{\text{Estrone} + \mathrm{TPNH} + \mathrm{H}^+ \rightleftharpoons \mathrm{TPN}^+ + \text{Estradiol}} \\ \text{Sum:} & \mathrm{DPN}^+ + \mathrm{TPNH} + \mathrm{H}^+ \rightleftharpoons \mathrm{TPN}^+ + \mathrm{DPNH} + \mathrm{H}^+ \end{array}$$

This formulation implies that the estrogen-sensitive transhydrogenase is the same enzyme as the estradiol-17β dehydrogenase (EβDH) described by Langer and Engel.[8]

Coupling of the Estrogen-Sensitive Enzyme with Glucose-6-Phosphate Dehydrogenase

It was readily possible to confirm Talalay's observation that estrogens stimulate glucose-6-phosphate dehydrogenation, as well as isocitric acid dehydrogenation (FIGURE 1).[9] The experiments were carried out using the protein precipitated from the nonparticulate fraction of placental homogenates (^{s}57,000) by 40 per cent saturation with ammonium sulfate. This protein was dissolved in 0.1 M Tris buffer, *p*H 7.4, and placed, together with 1.5 μM of DPN and 10 μM Mn^{++}, in the cuvettes of a Beckman spectrophotometer. Estradiol, in a concentration of 4×10^{-6} M, was added to one cuvette. After the reaction had proceeded for 20 min., a catalytic amount of TPN (0.075 μM) was added to both cuvettes. It can be seen (FIGURE 1a) that no reaction occurred in the absence of substrate and that essentially no reaction occurred in the presence of either substrate until TPN was added (FIGURES 1b and 1c). Upon the addition of TPN, a rapid reduction of pyridine nucleotide, measured as an increase in optical density at 340 mμ, was observed in the presence of estradiol with either isocitrate or glucose-6-phosphate as substrate.

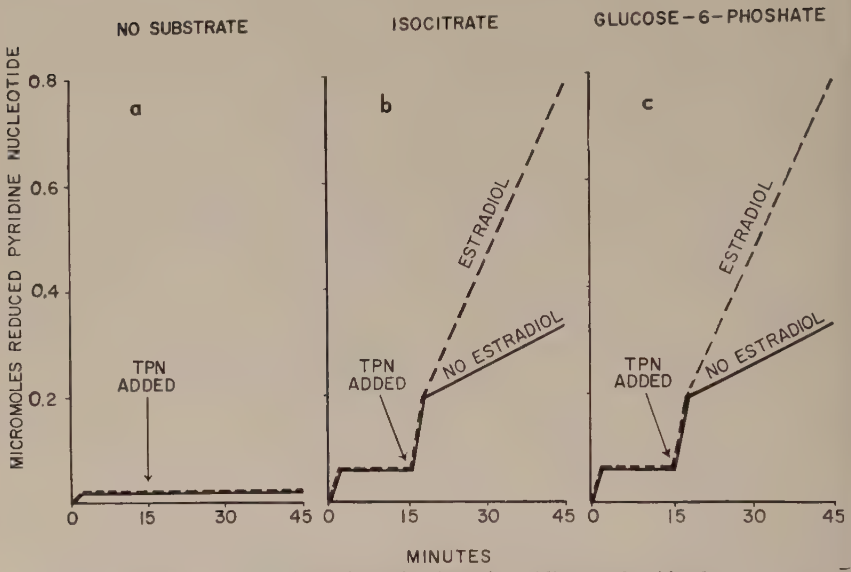

FIGURE 1. Effect of estradiol on the reduction of pyridine nucleotides by enzymes precipitated from placental $^{S}57{,}000$ preparation by addition of ammonium sulfate to 40 per cent saturation. Protein was redissolved in 0.1 M Tris buffer, *p*H 7.4. Each cuvette contained 1.0 ml. of protein in Tris buffer, 1.5 μM DPN, and 10 μM Mn^{++}. One cuvette of each pair contained estradiol, final concentration 4×10^{-6} M. FIGURE 1a shows results when no substrate was added; 1b, results when 6 μM of dl-isocitrate were added; and 1c, results when 3 μM of glucose-6-phosphate were added. The total volume in each cuvette was 3.0 ml.

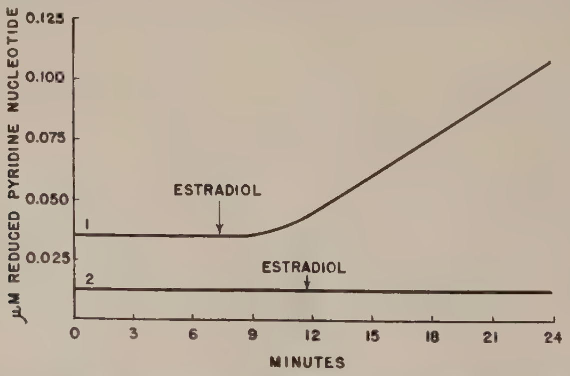

FIGURE 2. Transhydrogenase activity in the presence of yeast TPN isocitric dehydrogenase. Both cuvettes contained, in a total volume of 4.0 ml., 1.2 mM Tris buffer, *p*H 7.5, 10 μM MgCl$_2$, 4 μM DPN, 8 μM dl-isocitrate, and 0.2 ml. enzyme solution (30 per cent ammonium sulfate precipitate from $^{S}57{,}000$). In addition, cuvette 1 contained 0.1 ml. of the yeast enzyme and 0.1 μM TPN. Estradiol-17β was added at the times indicated to give a final concentration of 3×10^{-6} M. The curves are redrawn from a recorder plot.

The demonstration that the estrogen-sensitive enzyme requires a catalytic amount of TPN provides an explanation for our finding that there is an apparent requirement for ATP in our purified enzyme system, although ATP is not required for the estrogen stimulation of the crude nonparticulate fraction of the placenta.[4] The soluble protein fraction of the placenta apparently contains a phosphokinase that catalyzes the reaction:

$$DPN + ATP \rightarrow TPN + ADP$$

The conversion of a small amount of DPN to TPN would provide the necessary catalytic amount required for the transhydrogenase and would explain the observed conversion of ATP to ADP.

Coupling of the Estrogen-Sensitive Enzyme with Yeast Isocitric Dehydrogenase

In other experiments (FIGURE 2) a purified preparation[10] of the TPN isocitric dehydrogenase from yeast was used together with the fraction obtained from the placenta by 0 to 30 per cent saturation of the supernatant fraction ($^{s}57{,}000$) with ammonium sulfate. The control experiment (FIGURE 2, CURVE 2) shows that the latter has no DPN-linked isocitric dehydrogenase activity of its own. When the two enzyme preparations are mixed together in the presence of substrate amounts of DPN and catalytic amounts of TPN, an effect of estradiol n increasing the rate of reduction of DPN is readily demonstrable (FIGURE 2, CURVE 1). These experiments also support the hypothesis that estradiol activates a transhydrogenase rather than an isocitric dehydrogenase.

Effect of Estrogen on Glucose-1-C^{14} Oxidation

To study this reaction further by a method that does not involve the measurement of the reduction of pyridine nucleotide, we turned to the glucose-6-phosphate dehydrogenase system and glucose-1-C^{14} or glucose-6-C^{14}. The soluble protein fraction of the placenta ($^{s}57{,}000$), or the 0 to 40 per cent saturated ammonium sulfate fraction dissolved in Tris buffer, was incubated in Warburg vessels with glucose-1-C^{14} or glucose-6-C^{14}, hexokinase, 1.0 μM of ATP, 1.5 μM of DPN, with or without 0.075 μM of TPN and with or without estradiol, in a total volume of 3.0 ml. These experiments were carried out at *p*H 6.6, which appears to be the optimum *p*H for the estrogen-sensitive enzyme.

In the first experiments, yeast glucose-6-phosphate dehydrogenase was added, but this proved to be unnecessary and was omitted from later experiments. There is enough glucose-6-phosphate dehydrogenase present in the placental preparations so that this step is not rate limiting. The enzyme was tipped in rom the side arm of the flask at zero time. The center well of the vessel conained 5 per cent CO_2-free sodium hydroxide. After a 1-hour incubation at 37° C. the carbon dioxide produced was removed from the center well and precipitated as barium carbonate. The barium carbonate was transferred to tainless steel planchets and counted in a windowless proportional flow counter. Aliquots of the incubation medium were analyzed for total reducing substance by the method of Nelson.[11] Glucose from other aliquots of the incubation medium was precipitated as the phenyl osazone, recrystallized, and analyzed or its C^{14} content. Other aliquots of the incubation medium were placed in uvettes, and the amount of reduced pyridine nucleotide was measured in the pectrophotometer at 340 mμ. The results given in TABLE 1 provide further onfirmation of the hypothesis that estrogens affect a transhydrogenase for. lthough a slight estrogen stimulation is evident in the absence of added TPN,

TABLE 1

EFFECT OF ESTRADIOL ON THE METABOLISM OF GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY PLACENTAL PREPARATIONS

Additions to enzyme system, μM			Glucose-1-C^{14} converted to $C^{14}O_2$	Glucose-6-C^{14} converted to $C^{14}O_2$	Pyridine nucleotides reduced
DPN	TPN	Estradiol			
1.5	0	0	6 ± 0.7	2.3 ± 0.2	58 ± 7.7
1.5	0	0.011	11 ± 2.9	2.3 ± 0.2	81 ± 10.0
1.5	0.075	0	53 ± 5.5	2.2 ± 0.2	73 ± 5.6
1.5	0.075	0.011	120 ± 11.0	2.4 ± 0.2	128 ± 13.0

The figures represent mean ± S.E. of 10 determinations, expressed as millimicromoles per hour per vessel.

TABLE 2

EFFECT OF VARYING CONCENTRATIONS OF DPN AND TPN ON THE ESTROGEN-SENSITIVE ENZYME SYSTEM OF THE NONPARTICULATE FRACTION OF THE PLACENTA

DPN added, μM	TPN added, μM	α-Ketoglutarate produced, μM		Pyridine nucleotide reduced, μM	
		No estradiol	estradiol	No estradiol	estradiol
1.5	0	0.33	0.51	0.19	0.36
1.5	0.075	0.32	0.52	0.17	0.40
1.5	0.375	0.41	0.50	0.22	0.45
1.5	0.75	0.74	0.75	0.54	0.54
1.5	1.5	1.21	1.22	0.93	0.96
0	1.5	1.02	1.07	0.77	0.80
0	3.0	2.54	2.59	1.16	1.24

Each figure is the mean of 6 determinations.

a much greater one is evident when catalytic amounts of TPN are added. The carbon 1 of glucose is the one that appears as carbon dioxide as a result of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase activity. Both of these enzymes require TPN. The effect of estrogen in increasing the amount of C^{14} in the carbon dioxide given off is readily evident. However, estrogen had no effect on the conversion of carbon 6 of glucose to carbon dioxide.

Differentiation Between Transhydrogenase and Isocitric Dehydrogenase as the Site of Action of Estrogen

The fact that an estrogen stimulation of glucose-6-phosphate dehydrogenation can be demonstrated in the presence of catalytic amounts of TPN is strong evidence for the transhydrogenase theory. If the role of estrogen is to speed up transhydrogenation, that is, to convert TPNH and DPN to TPN and DPNH, then the estrogen effect should be obliterated in the presence of high concentrations of both DPN and TPN. It had been shown previously[4] that no estrogen effect was demonstrable in the presence of TPN alone. In contrast, if estrogen affects a DPN isocitric dehydrogenase directly, and if isocitrate concentration is not rate limiting, then an estrogen effect should be evident in he presence of DPN even when high concentrations of TPN are added. The xperiments summarized in TABLE 2, in which both pyridine nucleotide reduction and α-ketoglutarate production were measured, show that the former rediction is realized. This constitutes further evidence that a transhydroenase, not a DPN-linked isocitric dehydrogenase, is the enzyme most immeditely affected by added estrogen.

The Reversibility of the Transhydrogenase

If estrogens stimulate a transhydrogenase, and if the transhydrogenation eaction is readily reversible, then it should be possible to demonstrate a stimlation by estrogen of an enzyme that requires DPN and is supplied with subtrate amounts of TPN, catalytic amounts of DPN, and the fraction from the lacenta containing the transhydrogenase. This prediction was tested with oth lactic dehydrogenase and alcohol dehydrogenase. The nonparticulate raction of the placenta has been shown to contain lactic dehydrogenase.[6] amples of crystalline alcohol dehydrogenase from yeast and from liver were enerously provided by Bert Vallee.

It has not been possible so far to demonstrate an estrogen stimulation of actic dehydrogenase in either the forward direction (lactate + DPN$^+$ →

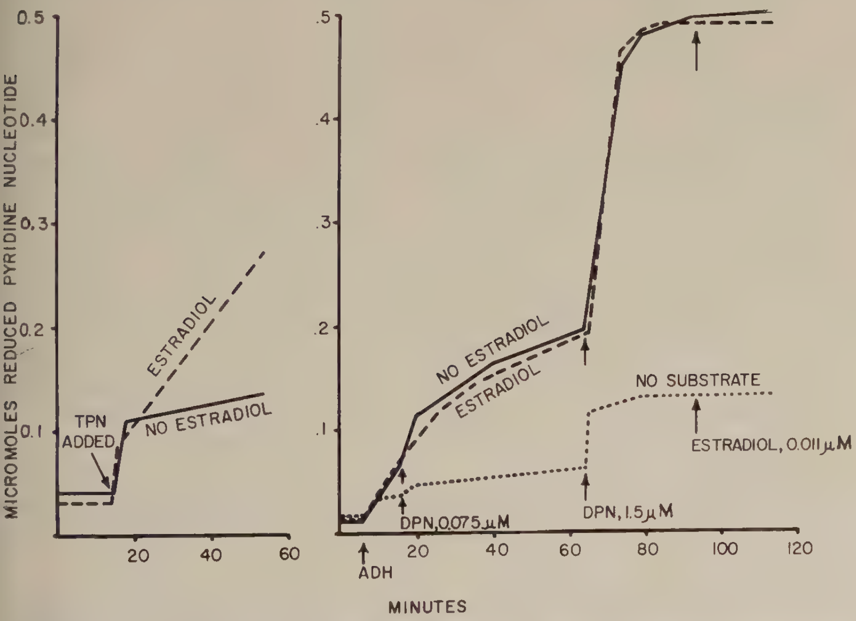

FIGURE 3. Effect of estradiol on reduction of pyridine nucleotides by enzymes precipitated rom placental S57,000 preparation by the addition of ammonium sulfate to 40 per cent saturaion. The curve at the left was a test of the enzyme preparation to show that transhydrogenase ctivity was present. The conditions were identical with FIGURE 1c; glucose-6-phosphate was he substrate. The curves on the right were obtained with the same enzyme preparation and vith ethanol as the substrate. At the times indicated, yeast alcohol dehydrogenase, 0.075 M of DPN, 1.5 μM of DPN, and 0.011 μM of estradiol were added.

pyruvate + DPNH + H^+) or in the reverse direction, nor has it been possible to demonstrate an estrogen stimulation of alcohol dehydrogenase.[9] Each sample of the transhydrogenase preparation from the placenta was tested for its ability to couple with glucose-6-phosphate dehydrogenase. All experiments were performed in the Beckman spectrophotometer, and the reaction was followed by measuring the optical density at 340 mμ. The preparations clearly contained lactic dehydrogenase or alcohol dehydrogenase activity, respectively, but neither one was significantly increased by the addition of either estradiol-17β or estrone (FIGURE 3). The curves in FIGURE 3 show that when substrate amounts of DPN are added later, pyridine nucleotide reduction occurs rapidly.

Discussion

In the normal cell most of the TPN is present in the reduced form, TPNH; in contrast, most of the DPN is present in the oxidized form, DPN^+.[12] There are several important oxidative reactions that require TPN^+. Under certain circumstances, the amount of TPN^+ present may be rate limiting; thus, a reaction that can increase the amount of TPN^+ would be very important in the over-all economy of the cell. A transhydrogenase, by increasing the amount of TPN^+ available, would increase the rate of reactions such as those of isocitric dehydrogenase and glucose-6-phosphate dehydrogenase, which are key reactions in the Krebs citric acid cycle and the hexose monophosphate shunt, respectively. This in turn would increase the amount of biologically useful energy available to the cell.

An estimate of the amount of stimulation of isocitric dehydrogenase obtained with estradiol was made by measuring the total amount of isocitric acid metabolized by mitochondrial enzymes and by nonparticulate enzymes in the presence and absence of estradiol.[13] The isocitric dehydrogenase in the mitochondria accounts for a rather small fraction of the total isocitric acid oxidized by the cell. The mitochondrial TPN enzyme accounts for 14 per cent and the mitochondrial DPN enzyme accounts for 6 per cent of the total isocitric acid oxidized by the estrogen-stimulated cell. The addition of estradiol will more than double the amount of isocitric acid dehydrogenated by the enzyme in the nonparticulate fraction. The increase brought about by estrogen stimulation accounts for approximately 45 per cent of the total amount of isocitric acid dehydrogenated by stimulated placental cells. Thus, the stimulation of the estrogen-sensitive enzyme can lead to a quantitatively important increase in the amount of substrate metabolized by the Krebs cycle. The increased amount of biologically useful energy can be used by the cells for growth, for protein and nucleic acid synthesis, for the imbibition of water, and for the other physiological effects of estrogen *in vivo*.

Previously we tested a variety of steroids and related substances for their estrogenic or antiestrogenic activity in this system.[14] On the basis of these measurements, the apparent dissociation constant, K_H, of the enzyme-steroid complex, and K_I, the apparent dissociation constant of the enzyme-inhibitor complex, were calculated.[15] It was pointed out at that time that the definition of K_H is identical in form with the definition of K_M, the classic Michaelis constant for the dissociation of an enzyme-substrate complex. If the estrogen exerts its effect as an activator of an inactive enzyme, as we had postulated, hen the definitions of K_H, K_I, and θ, and the deductions made from the numerical values of these constants remain valid. However, since the estrogen-sensitive enzyme appears to be a transhydrogenase rather than an isocitric dehydrogenase, the constants would apply to the transhydrogenase-steroid complex.

If the hypothesis that estradiol serves as a coenzyme of the transhydrogenase is correct, then the K_H, as defined, does become the K_M of the placental estradiol-17β dehydrogenase. The value for the K_H calculated from the amount of α-ketoglutarate produced will not necessarily be numerically identical to the K_M of the estradiol dehydrogenase. The K_H calculated is related to the rate constant of whichever of the following reactions is slowest and therefore is rate limiting:

$$\text{Isocitrate} + \text{DPN}^+ \rightleftharpoons \text{DPNH} + \text{H}^+ + \alpha\text{-ketoglutarate} + \text{CO}_2 \tag{1}$$

$$\text{DPN}^+ + \text{estradiol} \rightleftharpoons \text{DPNH} + \text{H}^+ + \text{estrone} \tag{2}$$

$$\text{TPNH} + \text{H}^+ + \text{estrone} \rightleftharpoons \text{TPN}^+ + \text{estradiol} \tag{3}$$

$$\text{TPN}^+ + \text{isocitrate} \rightleftharpoons \text{TPNH} + \text{H}^+ + \alpha\text{-ketoglutarate} + \text{CO}_2 \tag{4}$$

Direct measurements of the rates of these reactions under the conditions existing during the measurement of K_H show that the velocities of reactions 2 and 3 are of the same order of magnitude and that reaction 4 is much faster. The constants K_H, K_I, and θ are related to these rate constants, but no exact meaning has been assigned to them at present. However, this does not invalidate their use as arbitrary parameters for the comparison of various estrogens and antiestrogens.

A corollary of the hypothesis that estradiol is a coenzyme that is reversibly oxidized and reduced by the transhydrogenase is that estrogenic substances such as bisdehydrodoisynolic acid and stilbestrol should be substrates for the enzyme and be oxidized or reduced by it. Such a reaction seems improbable and suggests that our original hypothesis, that estrogen is bound to an enzyme and activates it, may be correct. The estrogenic activity of bisdehydrodoisynolic acid in the intact animal is also not satisfactorily explained by the oxidation-reduction hypothesis, unless one postulates that the compound is modified considerably before it becomes estrogenic. Distinguishing between the two mechanisms will require the separation of the transhydrogenase from estradiol-17β dehydrogenase, or proof of their identity. Such studies are in progress.

Transferring the site of action of estrogen from isocitric dehydrogenase to transhydrogenase does not vitiate the arguments previously presented[16] regarding the physiological importance of the estrogen-sensitive enzyme, but rather extends the estrogen effect to other TPN-requiring systems.

A specific requirement for TPNH has been shown for a number of reactions nvolved in the synthesis of fatty acids, steroids, and purines. Mueller[17] has shown that, when estrogens are injected into a castrated rat, a stimulation o the incorporation of labeled 1-carbon fragments into serine and of labele glycine into nucleic acids subsequently can be demonstrated *in vitro*. Becaus of the time interval between the injection of estrogen and the demonstrabl effect, Mueller concluded that the estrogens act by increasing the activities o the enzymes involved.

In view of the great preponderance in the cell of TPNH over TPN^+ and o DPN^+ over DPNH, it seems unlikely that TPNH would be rate limiting in these synthetic processes, so that an acceleration of the reverse direction o the reaction

$$TPNH + DPN^+ \rightleftharpoons DPNH + TPN^+$$

would not account for the stimulation of the synthesis of purines and of the other substances observed by Mueller. It would seem more likely that Mueller's observations could be explained in terms of an increased supply of biologically useful energy brought about by the increased oxidation permitted by the transhydrogenase operating in the forward direction.

Summary

Evidence has been presented that the estrogen-sensitive enzyme of the placenta, and presumably of the endometrium, is a transhydrogenase and not an isocitric dehydrogenase. The transhydrogenase can be coupled with yeast isocitric dehydrogenase or glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase. The prediction that it should be possible to demonstrate a stimulation by estrogen of an enzyme that requires DPN and is supplied with substrate amounts of TPN, catalytic amounts of DPN, and the transhydrogenase preparation from the placenta was tested experimentally. Both alcohol dehydrogenase and lactic dehydrogenase were tested, but no estrogen stimulation of their activity was demonstrable.

Estradiol, by stimulating the transhydrogenase, produces a quantitatively important increase in the amount of isocitrate metabolized by the cell. The present evidence, which suggests that estradiol stimulates a transhydrogenase rather than isocitric dehydrogenase, does not negate the arguments regarding the possible physiological importance of the estrogen-sensitive enzyme, but extends the estrogen effect to other TPN-requiring systems.

Acknowledgments

I express my appreciation for the many contributions to this work made by D. D. Hagerman and E. E. Gordon. I also thank Janet Loring, Anita Sarner, and Frederica Wellington for their technical assistance in carrying out the experiments described. Gifts of estrogens and analogues from Ernst Báràny, Lewis Engel, Guy Marrian, Gerald Mueller, and Albert Segaloff are gratefully acknowledged.

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Discussion

L. L. Engel (*Massachusetts General Hospital, and Harvard Medical School, oston, Mass.*): The section on biochemical and histochemical aspects of the terus provides much food for thought and has indicated the new and fruitful irections now being taken in endocrinological research. The description in iorphologic terms of changes induced by hormone administration are supplemented by the measurement of changes in the relative and absolute amounts f tissue constituents, both organic and inorganic. From this stage it is atural to proceed to a more dynamic examination of the levels of activity of nzyme systems that may be concerned with the product of the chemical nergy required for growth. The activities of different hormonal agents in ltering enzymatic potencies may be correlated to some extent with the biogical potencies of the compounds as measured by conventional parameters. n this area a closer union of biochemical and histochemical techniques doubtss would lead to a clearer understanding of the distribution of both chemical onstituents and enzymatic activities within the cell.

The final area discussed, the mechanism of hormonal action, is one that is apidly moving to the fore in endocrinological research. The uterus, which is so sensitive to specific stimulation by estrogen, provided the model system for the more detailed studies on an estrogen-sensitive enzyme system in the placenta. In this coupled-enzyme system, the estrone or estradiol seems to play the part of a coenzyme (or cosubstrate). These observations point the way toward an understanding of the means by which estrogens exert their growth-regulatory effects. Such speculations are most tempting, but it should be recalled that there are many compounds with high estrogenic activity that structurally are incapable of participating readily in such hydrogen transfer systems. Moreover, the fact that certain compounds highly estrogenic in one species are inactive or only weakly active in another also must be brought into the structure of any theory of estrogen action. The cumulative evidence therefore suggests more than one biochemical site of action of estrogen in the stimulation of uterine growth.

Part III. Problems in Uterine Tumors

ADENOCARCINOMA OF THE UTERINE FUNDUS IN THE RABBIT*

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The most common form of neoplasia in the domestic rabbit is an adenocarcinoma of the uterine fundus. Polson,[1] in a review in 1927, found reference to 29 instances and noted that approximately 50 per cent of the tumors reported were of this variety. Furthermore, in a systematic study involving a colony of approximately 850 female rabbits, more than 140 such tumors were found over a 9-year period.[2-4] The growths present features of interest in relation to development and incidence, and have been studied primarily from these points of view.

Clinical Course

Analysis of the histories of the 142 animals concerned in this report shows that a period of disturbance in reproductive function always precedes the detection of the tumor. Fertility is diminished, the litters born are reduced in size and contain many dead young, and desertion by the mother is common. In addition, other abnormalities of gestation, such as retention of litters *in utero*, abdominal pregnancy, abortion, or resorption of the products of conception frequently are encountered.

In general, the period of altered reproductive function extends for 4 to 6 months prior to the clinical detection of the tumor. Detection of the tumor depends on palpation of persistent uterine nodules; in this connection it should be noted that all of the animals were carefully examined at 10-day intervals. When first noted, the nodules approximate the size of 10-day pregnancy cysts. They are frequently multiple and present in both horns. In approximately one fourth of the cases cystic breast changes are observed coincidentally.

The growth rate of the tumors varies in different animals. In most instances they reach the size of hen's eggs in 6 months, but occasionally they remain without observable growth until death from metastasis. In general, the duration of the disease from clinical detection to metastasis averages 12 months. The breast lesions usually persist without change, but in a number of cases they have progressed to adenocarcinoma.

Pathology

The tumors arise from the glandular epithelium of the mucous membrane of the uterus and preserve an adenomatous structure throughout their course. Very early tumors are polypoid and are usually situated in the mucosal folds adjacent to the mesometrial insertion. In the majority of cases, they are

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multiple and spaced regularly throughout both horns. Histologically they are made up of irregular glandular elements embedded in a richly vascular myxoid stroma.

Advanced growths are associated with deep muscular invasion and extension laterally in the endometrium. Anaplasia with disorganization of architecture is the rule, but occasionally tumors show little histological change throughout their course. Rarely, the various centers of growth may remain discrete, but they usually coalesce by lateral extension and, with deep muscular invasion and growth of secondary nodules, the entire uterus is converted into a mass of tumor tissue.

Invasion of vascular walls and rupture into blood vessels is observed frequently in early clinical stages, but metastasis is delayed until late in the disease and is always associated with extensive necrosis of the primary tumors. It is widespread, and metastases have been found in all organs of the body. Dissemination also occurs by direct extension of the primary growth into the mesometrium, and diffuse infiltration of the pelvis and abdomen may antedate metastasis by several months.

Pathological changes rarely found in other conditions have been observed with high frequency in the thyroid and adrenal, but other organs show no consistent alteration. The lesions in the thyroid and adrenal are present from the earliest stages of the disease and have been found in animals sacrificed during the period of reproductive disturbance preceding the appearance of neoplasia.

The thyroid is small and is made up of tissue histologically resembling the fetal adenomas found in man. The alteration is diffuse, however, and usually no normal acini are found. The changes in the adrenals are most marked at the inner boundary of the zona fasciculata and resemble those observed in toxemia of pregnancy in the rabbit. The cells of the area contain large amounts of lipoid material and often coalesce to form rounded masses. Frequently the entire inner circumference is involved, and the process is so pronounced that the cortex and medulla are separated by a wide zone of more or less homogeneous pale staining material.

The appearance of the pituitary is not consistent, but generally there appears to be a deficiency of chromophilic cells and an increase in chromophobes. The intermediate zone is unusually wide and contains an abundance of colloid.

Incidence

The total incidence of the tumors in a group of 849 rabbits was 16.7 per cent, but it varied widely in relation to age, breed, and other constitutional factors.

The incidence increased from 4.2 per cent in animals 2 to 3 years old to 79.1 per cent in animals 5 to 6 years old. It should be noted that the figures given above represent new tumors occurring at the stated ages.

No instance of the tumor occurred in the Belgian or Rex breeds, and the arrangement of breeds in order of increasing incidence stands as follows: Polish, Himalayan, Sable, Beveren, Chinchilla, English, Marten, Dutch, Havana, French Silver, and Tan. The variation extended from 8.3 per cent n the Polish to 50 per cent in the Tans.

The total incidence in the inbred or comparatively homogeneous population was 14.2 per cent, in contrast to 21.1 per cent in hybrid stocks. This variaion is of considerable interest inasmuch as the pure breeds represented a oundation stock of normal animals, whereas the hybrids were maintained because they showed or transmitted constitutional variations. The influence of these variations on tumor incidence was most apparent in the case of transmitters of two endocrinological deficiencies manifest as dwarfism in one case and cretinism in the other.[5, 6] The incidence among transmitters of dwarfism was 48.2 per cent and of cretinism 33.7 per cent, in contrast to an incidence of 14 per cent in the remainder of the population.

Relation to Disorders of Pregnancy

A point of significance with reference to the etiology of the tumors concerns the similarity of variations in their incidence with those found to characterize a fatal rabbit disorder that resembles toxemia of pregnancy in humans.[7, 8] With few variations in position, the standing of the various breeds in susceptibility scales was essentially the same. Furthermore, the incidence of toxemia of pregnancy as well as of uterine tumors was highest in first-generation hybrids and in constitutional groups characterized by transmission of the dwarf and cretinoid abnormalities.

This close parallelism suggested a possible relationship between fatal toxemia and the disorders of pregnancy known to characterize the breeding history of animals that subsequently developed uterine tumors. Accordingly, an nvestigation of such poorly defined, nonfatal disorders was instituted. In brief, it was found that the chemical blood alterations and tissue changes were identical in type with those observed in fatal cases, and it was concluded that the disorders under consideration represented less acute disturbance of the same nature.

It is of interest that typical changes were found when the blood of a number of animals included in the present report was studied during attacks of the mild disorder several years prior to tumor development. In other instances, blood studies were not carried out, but a review of breeding records shows that all of the tumor-bearing rabbits suffered disorders with similar clinical signs at early periods of their history.

Relation to Endometrial Hyperplasia

The frequency of an antecedent history of toxemia of pregnancy in tumorbearing animals suggested that continued study after an attack might reveal significant preneoplastic changes in the endometrium. Accordingly, such nimals were subjected to monthly laparotomy and endometrial biopsy. In rief, early sections appeared normal but, in all cases, sequential biopsies howed the development of cystic hyperplasia that persisted throughout the remainder of the course. It is significant that all of the animals selected for tudy on a basis of an attack of toxemia of pregnancy and followed for 18 months developed typical uterine tumors, and that, in all instances, the development of the tumor was preceded by cystic endometrial hyperplasia.

It is of further significance that a number of animals sacrificed for more detailed study during the stage of endometrial hyperplasia showed the endocrinological changes characteristic of tumor-bearing animals.

Coincident Tumors

Fifty-three of the animals in the present series bore other primary tumors, and 45 of these were situated in the same organ system (7 leiomyomas and 5 leiomyosarcomas of the uterus, 14 papillomas, and 19 adenocarcinomas of the breast).[9] This finding is of interest in view of the endocrinological status of the animals and suggests that the factor concerned in the initiation of the adenomatous uterine tumors was also operative on other tissues of the reproductive system.

A further significant point with reference to the second neoplastic focus concerns its inhibitory effect on the progress of the other in its development to metastasizable cancer. It was repeatedly noted that, in the presence of a second neoplastic focus, the uterine tumor failed to attain metastasizability despite a duration far beyond that required for full development in other animals. Moreover, removal of the second focus was followed by rapid attainment of this property and by death from metastasis.

Biological Evolution

The routine performance of endometrial biopsies on animals after recovery from an attack of toxemia of pregnancy resulted in the discovery of many early tumors. In such cases, monthly biopsies were continued throughout the course of the tumors, and tissue fragments so obtained were subjected to experimental study. The primary object of the investigation was to compare the transplantability and growth rate of the tissue at different phases of development; to this end, representative fragments were transplanted autologously, homologously, and heterologously.[10]

In brief, it was found that, prior to the development of the ability to metastasize, tumor tissue would not survive transfer to normal animals. At this stage, however, autologous transfer or transfer to other animals bearing spontaneous uterine tumors resulted in growth. Transfer to normal animals became possible only after the tumor had attained the ability to metastasize and, at this stage, growth was obtained as readily in alien species as in the species of origin.

It was concluded that, prior to the development of metastasizability, the tumors were dependent on factors peculiar to the tumor-bearing animal. Such factors were not supplied by normal animals and, accordingly, the tumors would not survive transfer to normal animals. With continued development the tumor acquired the ability to metastasize; simultaneously, it attained independence of the factors essential to its earlier existence. The independence attained was such that species variations as well as individual constitutional differences were no longer barriers to transplantation, and growth occurred on transfer to alien species.

The nature of the factors concerned in the dependence of premetastasizable tumors is obscure, but experimental findings are indicative. It has been found that they are present from the earliest stages of tumor development and are systemic or constitutional in distribution. Animals with minute spontaneous tumors support growth of transplanted dependent tissue, and fragments of dependent tissue transplanted autologously in dispersed areas of the body attain autonomy simultaneously.

Further, the presence of identical endocrinological changes in all tumor-bearing animals suggested that the attendant systemic alteration might be the constitutional factor concerned, and the similarity of the changes with those induced with estrogenic hormone suggested the possibility that it might be the exciting agent. Accordingly, in a number of experiments rabbits subjected to long-continued treatment with this hormone were used as recipient hosts of tumor tissue shown to be in a dependent phase by transplantation tests. It is significant that in all instances the transplanted tissue survived and grew.

The endocrinological status of rabbits with spontaneous breast tumors is comparable with that of animals bearing adenomatous uterine tumors, and it provides the factors requisite for the growth of the latter tumors in dependent phase.[11] However, the essential factors are not provided by rabbits bearing spontaneous tumors of other organ systems, nor will dependent tumors of other organ systems survive transfer to animals with spontaneous uterine tumors. The specific relationship of the factors concerned in dependence is llustrated further by the fact that dependent tumors of the squamocolumnar junction of the vagina, although epidermoid in character, will survive transfer to animals bearing fundic tumors.

Carcinoma of the Squamocolumnar Junction

Carcinoma of the cervix has not been reported in the rabbit, nor has a single instance been noted in this laboratory despite careful search during autopsy of nearly 4000 females more than 2 years of age. The absence of a squamocolumnar junction in the rabbit's cervix suggests an anatomical basis for this observation. The columnar epithelium of the rabbit's fundus continues uninterruptedly over the cervix and down the vaginal wall to a junction with squamous epithelium at about the level of the urethral meatus; the occurrence of cancer at this location emphasizes the significance of the junction as a predisposing factor in carcinogenesis.[12]

The point of present interest relates to the fact that rabbits bearing carcinomas of this junction show endocrinological changes identical with those found in animals with spontaneous fundic tumors. Further, the two tumors occur in animals of the same constitutional type, and both are associated with an antecedent attack of toxemia of pregnancy.

Discussion

An investigation of adenocarcinoma of the uterine fundus in the rabbit brings out two main points that warrant emphasis. First, the incidence is highly selective, and susceptible animals are marked by distinctive constitutional traits. Second, the tumors are not endowed with the distinctive attributes of cancer from their inception. On the contrary, such attributes are developmental acquisitions, and their evolution depends on the continued operation of specific factors.

The features distinguishing susceptible animals appear to be genetic in origin and are associated with breed and with heterozygosity for various constitutional deficiencies of an endocrinological order. The role of such genetic factors in determining susceptibility is not apparent, and the relationship seems not to be direct but to depend on a causal sequence in which an intermediate effect is of great importance. The close parallelism with the incidence of toxemia of pregnancy is highly suggestive in this respect. Analysis of this disorder shows a similar frequency distribution with the same breed and constitutional relationships; moreover, there is definite evidence that all of the tumor-bearing animals suffered mild attacks of toxemia during earlier life.

It is suggested, accordingly, that the constitutional relationships observed in a study of the incidence of uterine tumors rest primarily on susceptibility to toxemia of pregnancy and gain expression through this circumstance rather than through the action of independent determining factors.

The mild form of toxemia suffered by the animals has been studied intensively and has been shown to be characterized by the same chemical and histological changes found in the fatal disorder. The most extensive damage occurs in the liver, and there is evidence from chemical blood study as well as from histological examination that damage may persist for as long as a year after clinical recovery. It has been shown experimentally that the liver is the principal organ of the body concerned with the inactivation of estrogenic hormone and that damage renders it incapable of performing this function.[13-16] The endocrinological changes present in tumor-bearing animals are characteristic of those induced by estrogenic hormone. It is suggested that such changes are incident to an excessive concentration of this hormone resulting from an impairment of liver function relative to inactivation. In view of the known carcinogenic action of estrogenic hormone, it is not unreasonable to suggest further that the uterine tumors may arise on this basis and represent a natural analogue to the experimental induction of neoplasia with this substance.

The results of an experimental study bear directly on this suggestion. Nonfatal attacks of toxemia of pregnancy were induced in a group of six-month-old rabbits, and small doses of estrogenic hormone were administered weekly throughout the next three months.[17] A control group of normal, nontreated animals of the same age received a corresponding amount of estrogenic hormone over the same period of time. The endometrium of animals subjected to preliminary liver damage was extremely irregular, with ronounced cystic hyperplasia and small areas of early tumor formation, vhereas that of the control rabbits receiving estrogen alone showed only a noderate hyperplasia.

It would appear also that development of the uterine tumors to cancer lepends on estrogenic hormone or the constitutional status incident to its ontinued action. Prior to the development of metastasizability, the special onstitutional status characteristic of tumor-bearing animals is an essential equisite for their continued existence. Transfer to normal animals always ails, while transfer back elsewhere in the primary host or to other animals earing spontaneous uterine tumors invariably succeeds. Growth in the atter cases depends on the constitution of the recipient rather than on the resence of a uterine tumor, for takes also follow transfer to animals of similar onstitutional status bearing tumors of the breast or squamocolumnar junction, vhile transfer to constitutionally different animals bearing tumors of other rgans fails. Furthermore, in a series of transplantation experiments it was ound that uterine tumors shown to be dependent by transfer to normal anmals could be transplanted successfully to rabbits subjected to the long-ontinued administration of estrogenic hormone in small doses.

The dependent phase of development extends throughout the greater part f the tumor's course and terminates with the attainment of metastasizability. oincident with the acquisition of this property, the tumors attain indeendence of the specific factors previously essential for their continued existence nd will survive and grow in their absence. Transplantation to normal nimals becomes possible, and species is no barrier. It should be noted that he simultaneous attainment of metastasizability and autonomy is a constant eature in the development of all tumors studied in this laboratory and is ot a peculiarity characteristic only of the uterine tumors under discussion.

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ARCINOMA OF THE UTERINE CERVIX AND UPPER VAGINA: NDUCTION UNDER EXPERIMENTAL CONDITIONS IN MICE*

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Cancers of the uterine cervix and uterus contributed 8.2 per cent of all ncer deaths in the United States in 1948, or a total of 17,120 deaths.[1] It as estimated at that time that 20 per cent of all uterine cancers were cured d hence would not appear among the mortality figures. Because uterine ncer is sex-limited, only approximately half of the population being sus- ptible, the mortality figures based on eligibility for the disease would be .4 per cent in contrast to 8.2 per cent, and 12.6 per cent of deaths attributable cancer of the intestine and stomach, respectively. Only cancer of the ammary glands, based on a similar correction for its nearly sex-limited pearance, is a more frequent cause of death attributable to neoplasia.

The high incidence of uterine cancer is unique to man; few spontaneous ncers occur in animals of other species and, when they do occur, they arise ore frequently in the uterus than in the uterine cervix.

Subsequent to the demonstration of the growth-stimulating effects of rmones on the uterus and the identification of the estrogenic hormones, it as assumed that hormones might induce uterine or uterine cervical cancer.

The observations on uterine or uterine cervical cancer reported by a number investigators have been summarized recently[2, 3] and will not be given tailed review here. Procedures that induce prolonged estrogenic stimulation modifying the intrinsic hormone balances have led to the production of perplastic uterine growths in guinea pigs, rats, or mice and, in rabbits, to ncer of the uterus. Rabbits of a particular hybrid group were subject to a ique glandular syndrome and exhibited, among other things, endometrial perplasia, dependent neoplasia and, finally, cancer.[4-6] The injection of ogenous estrogens over prolonged periods of time reportedly has induced erine cancers in rabbits, but not in animals of other species, such as monkeys, gs, rats, guinea pigs, or mice. Fibromyomatous lesions occur in estrogen- eated guinea pigs,[7] and subserosal cancerlike lesions have appeared in uteri estrogen-treated multiparous mice.[8] Cancer of the uterine cervix, vaginal rnices, or upper vagina occurs infrequently in animals of most species. ice of one strain occasionally had carcinomas involving the uterine cervix or per vagina;[9] 5 of 56 mice observed until death had epidermoid carcinomas necropsy. The uterus and uterine cervical tissues of mice of other strains n become cancerous under suitable conditions. When small crystals of ethylcholanthrene were placed in contact with small pieces of uterus or of rvix of young mice, and these tissues were transplanted subcutaneously in

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related hosts, cancers formed within a few months.[10] Prolonged periods c estrogen treatment also induced uterine cervical cancer in mice of a numbe of strains, but tumors of the endometrium have not or have only rarely appeared.[11, 12]

The incidence of invasive epithelial lesions of the uterine cervix or uppe vagina of estrogen-treated mice has never been high unless the animals received prolonged periods of treatment. Attempts have been made to augmen the incidence of tumors in these areas by the topical applications of hormone and to study the effects of the chronic application of other substances o mixtures of substances.

Materials and Methods

Mice of 5 different inbred strains (BC, C57, A, CBA, and C3H*) were used for the experiments undertaken. Most of the mice were placed on experiment when 6 months old or older, and had been used previously in the colony for breeding stock (see TABLES 1 to 6). They were all from animals born and raised in the colony, and their parents and siblings were among animals observed throughout life on different experiments or as breeding stock. All matings were brother-sister. Several sublines of the BC strain have been maintained for several generations, but are not reported separately here.

The mice were vaccinated routinely† when 40 to 50 days old,[13] housed in wooden boxes 6 × 12 × 6 inches, usually 2 females and 1 male in each box for the breeding stock and 5 to 6 mice per box when assigned to experiments. Food (Purina Laboratory Chow) and water were available at all times. Softwood sawdust covered the floors of the boxes and was changed once or twice each week.

Several different methods were used for the intravaginal application of the hormones or other materials. Stilbestrol in oily solution was applied in quantities of 1 to 4 μg. 3 times weekly by intravaginal injection through a blunted and polished 22 gauge needle attached to a 1-cc. tuberculin syringe. The stilbestrol was dissolved in sesame oil, 100 μg/cc. The vaginal responses were followed among groups of mice by examination of exfoliated cells. All mice so treated were ovariectomized shortly before injections were started (TABLE 6).

Pellets of stilbestrol-cholesterol‡ (1 part to 3 parts) measuring approximately 5.0 × 1.7 mm. were made by a mold or press similar to the one described by Forbes.[14] The chemicals were mixed thoroughly by grinding in a mortar prior to being pressed into pellets. The pellets were attached to Nylon

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* Mice of all strains except the BC strain were derived from the colony maintained by L. C. Strong. They have been maintained for from 6 to 12 years as independent lines by brother-sister matings. The origin of the BC strain is described in *Cancer Research*, volume 14, pages 205 to 209, 1954.

† Vaccine virus was obtained from Ely Lilly and Company, Indianapolis, Ind.

‡ The stilbestrol was supplied by E. R. Squibb & Sons, New York, N. Y. (Diethylstilbestrol). The cholesterol U.S.P. XIII (cholesterone C.P.) was obtained from Eimer & Amend Co., New York, N. Y.

TABLE 1

(A) UTERINE CERVICAL AND VAGINAL LESIONS IN MICE OF SEVERAL STRAINS BEARING INTRAVAGINAL PELLETS OF STILBESTROL-CHOLESTEROL
(1 PART TO 3 PARTS, ±7 mg.)

Strain	Number of mice	Age started		Period treated		Age at death		Pellets*	
		Range (days)	Mean (days)	Range (days)	Mean (days)	Range (days)	Mean (days)	+	−
BC	40	130–452	341	90–424	263	390–811	605	17	23
C57	8	378–524	442	64–321	168	442–731	609	2	6
A	3	204–399	320	44–204	106	248–603	427	1	2
CBA	2	371	371	42–109	75	413–480	446	1	1
C3H	1		378		46		424	1	

* + Pellet found at necropsy; − no pellet found at necropsy.

(B) LESIONS AND THE AGE AT WHICH THEY WERE OBSERVED

	Uterine-cervical or vaginal				Subserosal	Pellet	
						+	−
Stage	1	2	3	4			
Number	4	2	7	8	1	15	5
Range	42–249	258–315	183–288	205–356			
Average	186	286	241	260	245		

threads* by a tie and, subsequently, both were coated by several dips in 1 per cent collodion. The collodion was added, not only to improve the security of the attachment, but to decrease the rate of absorption from the pellet. The pellets were placed in the upper vagina by attaching the thread to a straight needle extended through the vagina and cervix and passed through the lower part of one uterine horn, usually the right. The thread was secured after the pellet had been pulled into contact with the cervix. At first the thread was only knotted. Because many pellets so secured were lost, the thread was subsequently tied around the uterine horn. Fifty-four mice of the 5 different strains were so treated (TABLE 1). Pellets of estrone similarly prepared were placed in the upper vagina of ninteen mice.

With nineteen mice from 4 inbred strains, pellets of stilbestrol-testosterone propionate† similarly prepared were placed in the upper vaginal areas (TABLE 2). With two mice, pellets of progesterone similarly prepared were placed in the vaginas, and 12 mice were given intravaginally small pellets of methylcholanthrene similarly prepared.

Sixty-five untreated control mice set aside during the same period were followed throughout their life. In addition, 12 mice received pellets of col-

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* Size A Nylon 302, made by Hemingway & Bartlett Manufacturing Company, New York, N. Y.

† The progesterone and estradiol benzoate were supplied by CIBA Pharmaceutical Products Inc., Summit, N. J., either as crystals or in solution. The testosterone propionate was upplied by Organon Inc., Orange, N. J.

TABLE 2

SUMMARY OF OBSERVATIONS ON UTERINE CERVICAL AND VAGINAL CANCERS IN MICE BEARIN INTRAVAGINAL PELLETS OF STILBESTROL-CHOLESTEROL (SC)-TESTOSTERONE PROPIONATE (1 PART TO 3 PARTS), OF PROGESTERONE OR OF METHYLCHOLANTHRENE

Treatment	Strain	Number of mice	Age started		Period treated		Age at death		Tumors
			Range (days)	Mean (days)	Range (days)	Mean (days)	Range (days)	Mean (days)	
SP	BC	13	313–470	405	178–365	253	563–758	664	1 (1); 5 (3)
	C57	4		381	181–280	252		562	
	CBA	1		471		203		674	
	A	1		480		96		576	
Progesterone	BC	2	388	388	174–235	205	562–623	592	
Methylcholanthrene	BC	11	132–231	186	140–373	327	316–581	513	
	A	1		238		281		519	

TABLE 3

SUMMARY OF OBSERVATIONS ON UNTREATED MICE OR MICE BEARING PELLETS OF COLLODION OR OF CHOLESTEROL

Experimental group	Strain	Number of mice	Age at death		Age started		Period treated		Tumors
			Range (days)	Mean (days)	Range (days)	Mean (days)	Range (days)	Mean (days)	
Control	BC	43	401–892	666					1 (4)
	C57	9	480–632	630					0
	A	13	249–632	492					0
Collodion pellets	BC	12	313–731	533	130–413	283	168–372	250	0
Cholesterol pellets	BC	30	453–761	589	130–444	322	93–371	267	1 (1–)
	A	3	588–726	653	354–418	393	181–308	260	0
	C57	2	666–724	695	318–478	428	246–288	267	0

lodion intravaginally, and 35 mice were given pellets of compressed cholesterol prepared in the manner already described (TABLE 3).

Stilbestrol dissolved in 0.05 ml. of sesame oil was injected subcutaneously into 19 mice at the rate of 250 μg. weekly. Twenty-two mice received weekly subcutaneous injections of 16.6 μg. of estradiol benzoate dissolved in 0.05 ml. of sesame oil. Pellets of stilbestrol and cholesterol (1 part to 3 parts) weighing from 9 to 10 mg. were implanted subcutaneously in 9 other mice (TABLE 4).

Younger mice (74 to 126 days of age) were used to study the effect of prolonged application of materials under consideration for use as possible constituents of intravaginal contraceptives (TABLE 5). Powders prepared from ground pellets of these materials were applied intravaginally three times weekly. Applications of the powders were made through small "eye droppers." A small amount of the powder was placed in the tip of the pipette and delivered

TABLE 4

SUMMARY OF OBSERVATIONS ON UTERINE CERVICAL OR VAGINAL TUMORS IN MICE GIVEN ESTROGENS BY SUBCUTANEOUS APPLICATION BEGINNING AT AGES OF SIX MONTHS OR MORE

Estrogen used and method of application	Strain	Number of mice	Age started		Period treated		Age stopped		Tumors
			Range (days)	Mean (days)	Range (days)	Mean (days)	Range (days)	Mean (days)	
SS 250 µg.	BC	14	183–416	358	176–285	233	396–661	591	3 (1); 2 (2); 1 (4)
SS 250 µg.	CBA	1		260		266		526	0
SS 250 µg.	C3H	2	269–361	315		53	323–413	368	0
SC 7–8 mg.	BC	8	368–467	391	60–355	157	419–697	547	1 (1)
	C57	1		451		53		504	
EB 16.6 µg.	BC	15	285–505	357	28–273	190	313–620	549	4 (1); 3 (3); 1 (4)
	C57	6	380–500	420	80–224	141	471–604	563	0
	A	1		452		112		564	0

TABLE 5

A) SUMMARY OF OBSERVATIONS ON UTERINE CERVICAL AND VAGINAL CANCER AMONG MICE OF THE BC STRAIN GIVEN INTRAVAGINAL APPLICATIONS OF BASE* 3 TIMES WEEKLY BEGINNING AT AGES RANGING FROM 74 TO 126 DAYS (AVERAGE 100 DAYS)

	Total number	Age at death	
		Range (days)	Mean (days)
Mice with lesions	21	552–724	640
Mice without lesions	14	343–753	614
Total	35	343–753	620

(B) LESIONS AND AGE AT WHICH THEY WERE OBSERVED

Stage	1	2	3	4
Number	5	2	2	10
Range (days)	469–579	526–615	512–524	426–631
Average (days)	527	570	518	535

* The preparation designated "base" was a whitish light-weight powder containing urea 97.3 per cent), adipic acid (0.6 per cent), and carboxymethylcellulose acids (2.1 per cent), upplied by Ortho Research Foundation, Raritan, N. J.

nto the vagina. The average application weighed 10 mg. for the base (see below), individual applications ranging from 6 to 18 mg. The average application of the mixture weighed 7.3 mg., and ranged from 1.4 to 12.4 mg. Other mice received small pellets averaging 11.8 mg., and ranging from 7.6 o 18.0 mg., which were cut from larger pellets and placed in the vagina three imes weekly by small forceps.

The substances tested were mixtures, one containing urea (97.3 per cent), dipic acid (0.6 per cent), and carboxymethylcellulose acids (2.1 per cent).

TABLE 6

SUMMARY OF OBSERVATIONS ON UTERINE CERVICAL AND VAGINAL RESPONSES OF OVARIECTOMIZED MICE GIVEN INTRAVAGINAL APPLICATION OF 1 TO 4 μG. OF STILBESTROL DISSOLVED IN SESAME OIL 3 TIMES WEEKLY

Treatment	Strain and group	Number of mice	Age started		Period treated		Age at death		Tumors
			Range (days)	Mean (days)	Range (days)	Mean (days)	Range (days)	Mean (days)	
1 μg.	BC	9	237–539	346	201–492	323	483–776	669	2 (1); 1 (4)
	C57	1		300		305		655	
2 μg.	BC	3	178–291	216	151–425	325	329–653	540	1 (1); 1 (4)
	C57	2		350	437–475	456	787–825	806	1 (1)
2 μg.	BC	2	320–349	334	288	288	608–638	623	—
2 + 4 μg.	BC	3	250–296	265	477–429	478	729–773	743	1 (1); 1 (4)
1 + 2 μg.	BC	1		118		497		615	—

This mixture is designated base in TABLE 5. The second mixture contained phenylquinone (0.5 per cent), urea (96.9 per cent), adipic acid (0.6 per cent), and carboxymethylcellulose acid (2.0 per cent), and is designated mixture.*

The mice were sacrificed when their general health declined or when tumors were detected. At necropsy all organs were inspected, special care in all instances being given to the genital tissues. The vaginas were opened ventrally and inspected for irregularities of the mucosa. Colored photographs were made of many specimens. The tissues were fixed routinely in Bouin's fluid and stained in hematoxylin and triosine or eosin blue. The vaginal and cervical areas usually were sectioned serially and, unless large tumors were present, 2 of every 12 sections were mounted and stained for examination. Several test sections were cut from the paraffin blocks of the large tumors.

Tumors of organs or tissues other than the genital tissues also were studied.

Observations

Three of the 21 mice given small applications of stilbestrol intravaginally had cancers of the uterine cervix or upper vagina (lesions rated as stage 4 in TABLE 6). All of these mice, from the BC strain, were from 178 to 291 days old when treatment was started and, prior to being placed on experiment, had had either 1 or 2 pregnancies. The average period of treatment was 450 days, ranging from 398 to 477 days. The largest tumor, located in the dorsal wall of the vagina, involved the cervix and vaginal fornix, but apparently arose from the dorsal vaginal wall. It measured 6 × 10 mm., presented an irregular surface of eroded appearance (FIGURE 1), was broadly attached at the base, and had infiltrated through the wall of the vagina (FIGURE 2). The cells were arranged irregularly and showed few areas of cornification.

A second tumor, measuring 5 × 6 mm., arose from the distal vagina at the margin with the perineum. It was an epidermoid pedunculated growth. Several other small infiltrative lesions occurred in the thickened vaginal

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* The materials designated base and mixture were supplied by the Ortho Research Foundation, Raritan, N. J.

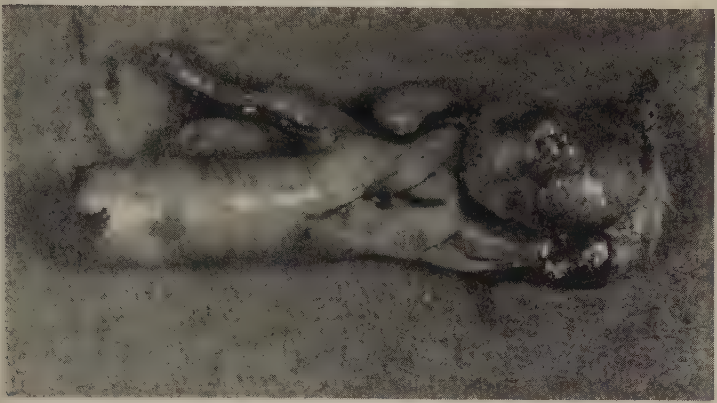

FIGURE 1. Photograph of the 2 uterine horns, cervix, and vagina of a mouse that had received applications of 2 or 4 μg. of stilbestrol intravaginally 3 times weekly for 477 days, beginning at 296 days of age. The epidermoid carcinoma filled the greater part of the vagina, which was opened ventrally to expose the tumor. The cervix and right uterine horn were involved in a reticulum cell carcinoma.

wall, one near the vaginal fornix and one on the dorsal vaginal wall. These smaller lesions were designated stage 2 (FIGURE 3).

The third large tumor involved about half the cervix of a mouse and apparently had arisen in the vaginal fornix and extended through the vaginal wall (FIGURE 4).

Small stage-1 lesions appeared in 5 other mice of this group after periods of treatment ranging from 151 to 492 days—an average of 370 days.

The condition of the vaginal mucosa was followed by obtaining smears of exfoliated cells for periods of about 3 months after the applications of hormone vere started. Most of the mice on all doses showed smears containing cornified cells for the first month. Thereafter the smears were variable, some animals tending to show more estrous smears than others. The response of the vaginal epithelium during the period studied did not reveal a predisposition for subsequent tumoral changes.

The vaginas nontumorous at the end of the experiment were all subestrous n the type of mucosal development shown. The epithelium, although stratfied squamous in type, was rarely as thick as at estrus and, when cornified, vas usually irregularly so. Adjacent areas seemed to differ greatly in structure. The uterine cervices were never as large as those from animals given estrogens systemically for prolonged periods. The uterine horns were rather vell developed, much as might be expected after prolonged periods of exposure o small amounts of estrogens.

Four mice of this group had lung adenomas; 3 had lymphomas; 2 had eticulum-cell sarcomas, in both instances involving the uterus and uterine ervix (FIGURE 5); and 1 mouse had a mammary adenocarcinoma.

Eight of 40 mice of the BC strain bearing intravaginal pellets of stilbestrol-holesterol had epidermoid carcinomas of the upper vagina or cervix (TABLE

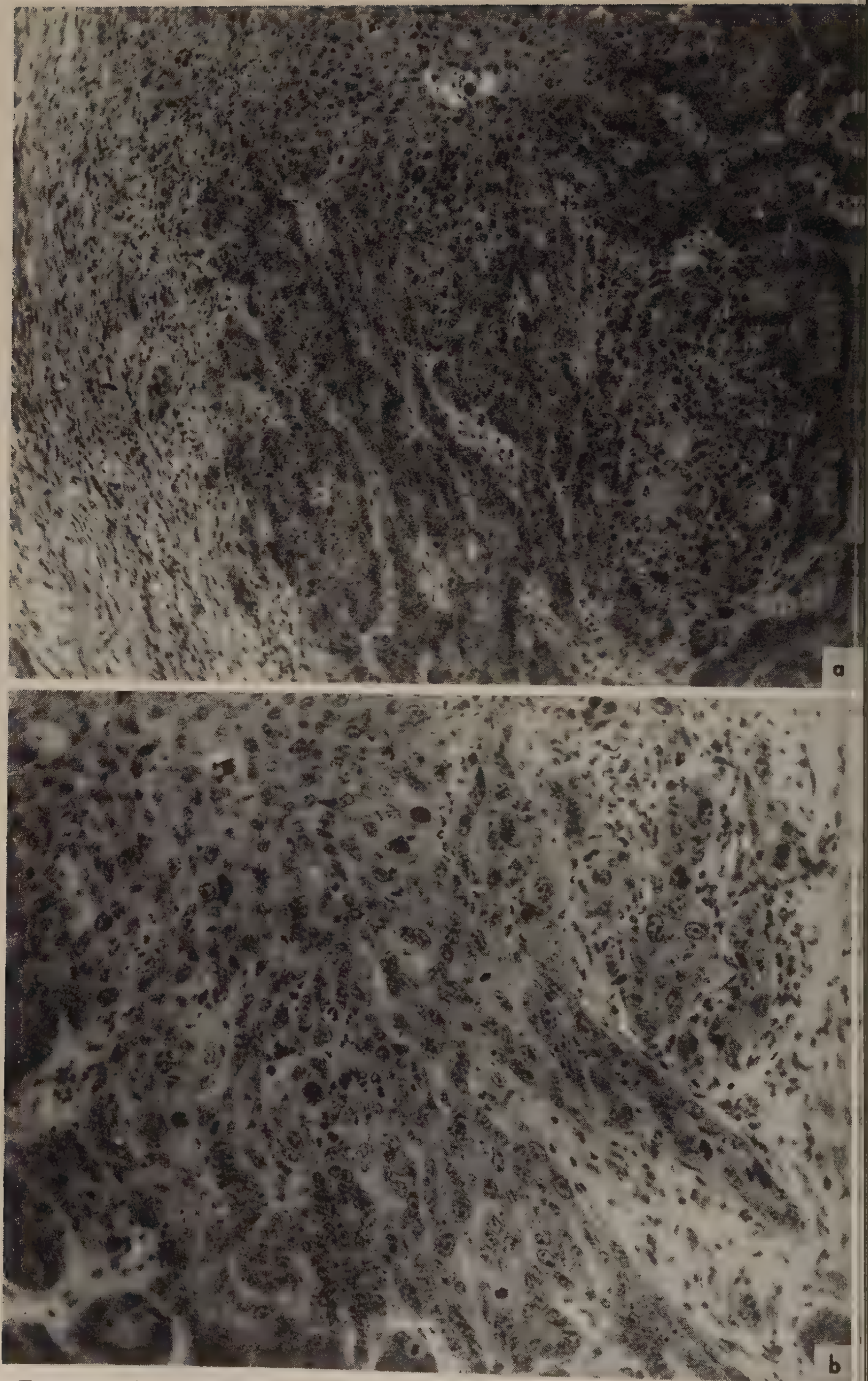

FIGURE 2. (a) Photomicrograph of the epidermoid carcinoma shown in FIGURE 1 (×120); (b) photomicrograph of the basal area of the same carcinoma. (×240).

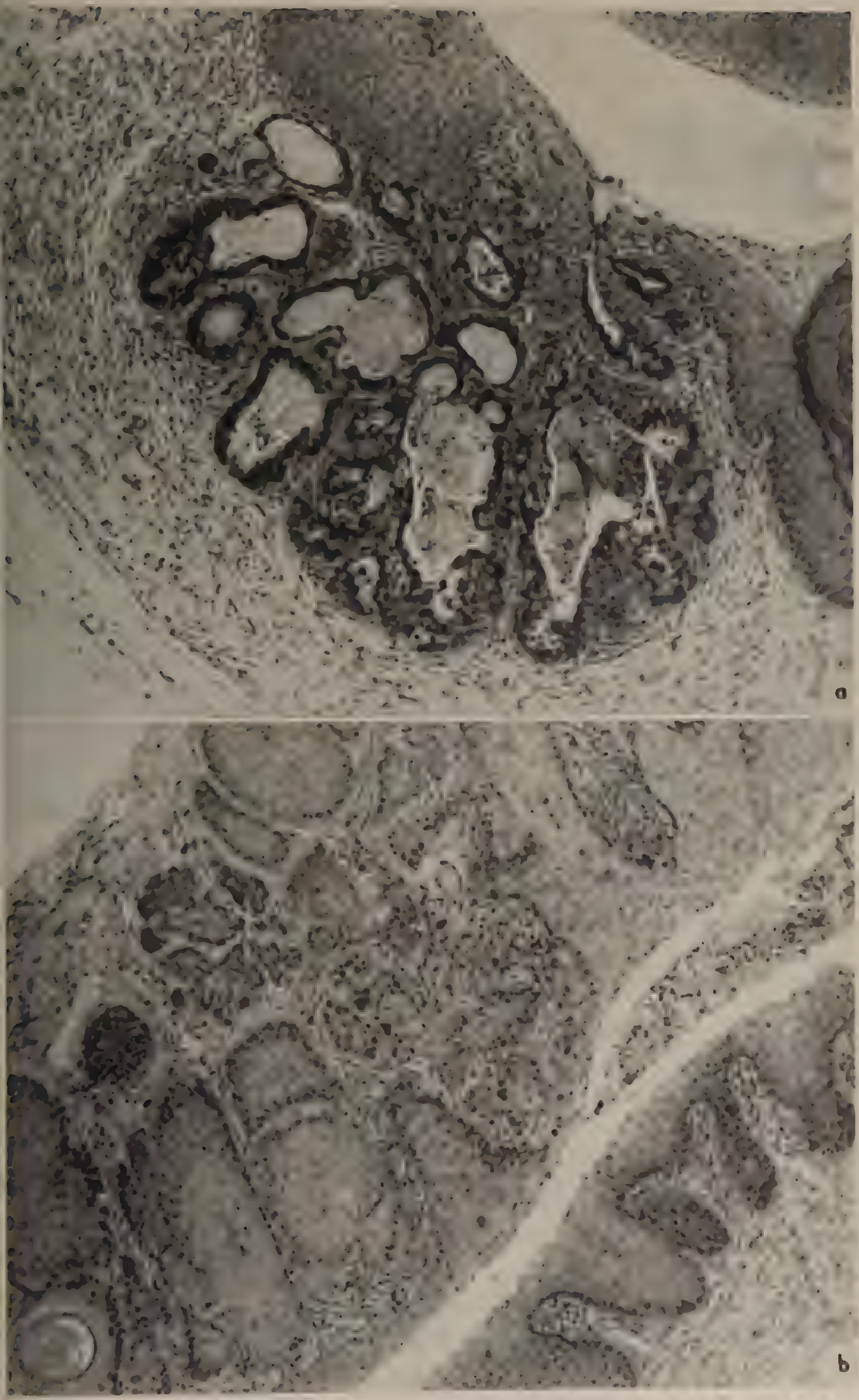

FIGURE 3. (a) Photomicrograph of a small lesion in a vaginal fornix of a mouse that had received 2 μg. of stilbestrol 3 times weekly for 475 days, beginning at 178 days of age (×120); (b) photomicrograph of a second small hyperplastic lesion in the upper vagina of the same mouse. (×120).

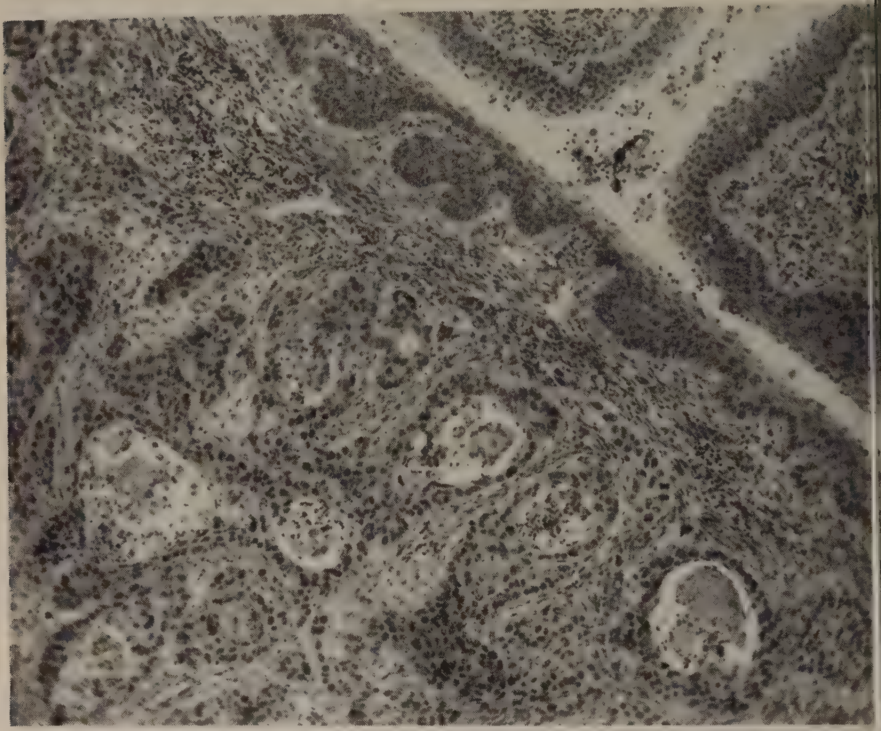

FIGURE 4. Photomicrograph of a portion of a stage-4 lesion of the vaginal fornix of a mouse that had received 1 μg. of stilbestrol 3 times weekly for 398 days, beginning at 291 days of age. ×120.

1), and 7 mice had lesions rated as stage 3 (lesions extending through the vaginal wall, but apparently not involving adjacent tissues[12]). The average period of treatment for the mice with the larger lesions was 260 days (TABLE 1B). All of these mice had pellets in the vagina at the time of necropsy. The pellets were not found at necropsy in 23 of the 40 mice of the BC strain. How long they had remained after being attached was unknown. Although the presence of the pellet was checked periodically, usually at 2-week intervals early in these experiments, the procedure was discontinued because the inspection itself tended to dislodge the pellet. It is undoubtedly significant that 15 of 17 mice that retained their pellets until necropsy had lesions. Unfortunately it is impossible to be certain how long pellets were retained by the other mice. The uteri and vaginal mucosae of the mice without pellets were usually atrophic, indicating that pellets had not been present for some time. When the pellets were in place at necropsy, the uteri always showed evidence of estrogenic stimulation. The uteri of some animals were cystic, others had uteri lined by a stratified squamous epithelium, and others showed partial epithelial metaplasia and spotty pyometra (FIGURES 6–9).

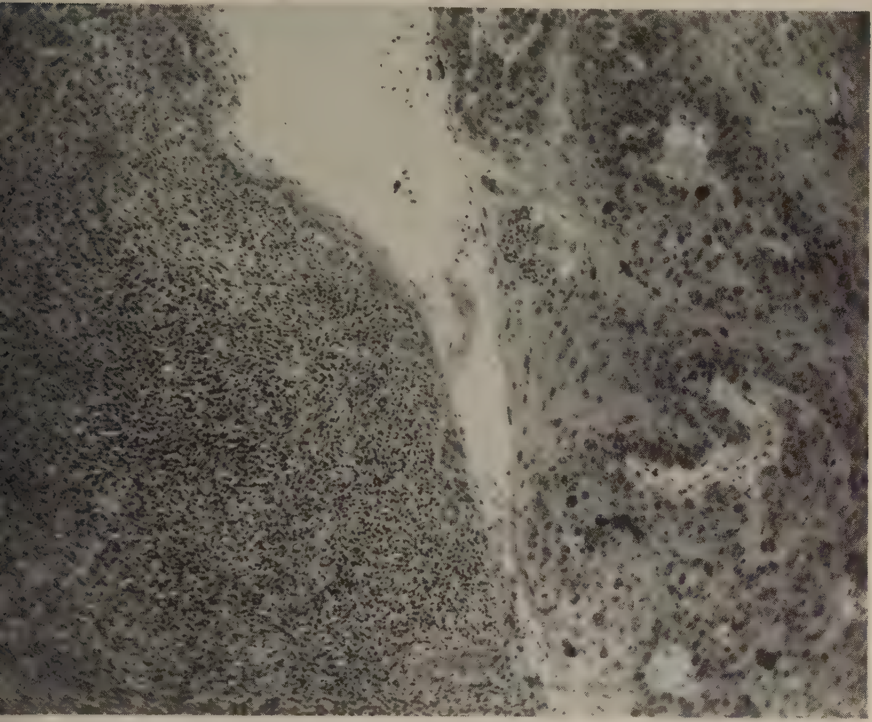

FIGURE 5. Section through the right vaginal fornix of the mouse shown in FIGURE 1, howing the reticulum cell sarcoma on the left and the epidermoid carcinoma on the right. ×120.

The estrogen was absorbed in amounts adequate to incite systemic effects s shown by the coexisting hypertrophy of the pituitary gland. Two mice ad pituitary chromophobe adenomas after carrying pellets for 266 and 290 lays. Other mice with enlarged glands showed a generalized degranulation f the chromophobic cells and diffuse hypertrophy of agranular cells.

The smaller invasive lesions were not unlike those described earlier and vere not infrequently multiple. They were randomly distributed in the pper vagina and vaginal fornices. None involved the external cervical *os*.

The larger lesions usually were detected some time before necropsy. Vaginal leeding was a frequent sign. Sometimes pelvic distension was noted as vell. A few epidermoid carcinomas or invasive epithelial lesions coexisted in nice with sarcomas of the reticulum-cell type. In one mouse of the BC train, sacrificed 286 days after the pellet was implanted, a large reticular ell tumor replaced the entire uterine cervix. Such tumors not infrequently ccur in untreated mice of the BC strain.

The failure of uterine cervical cancers to occur in mice of the C57, A, or BA strain, when similarly treated, does not mean that they are particularly

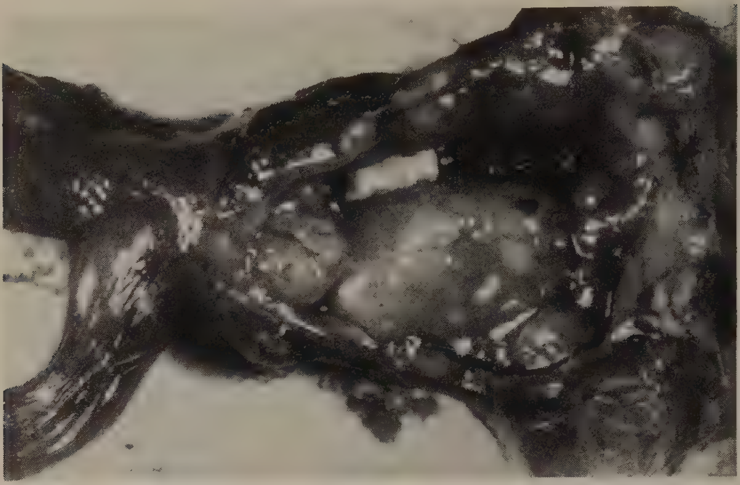

FIGURE 6. Photograph of the opened vagina, the cervix, and parts of the uterine horns of a mouse that had received a pellet of stilbestrol-cholesterol 310 days earlier, beginning at 382 days of age. The white pellet was in place at necropsy. The tumor involved the cervix and dorsal vaginal wall.

resistant to tumors of this type. The average survival period was less than that of mice of the BC strain by about 100 days or more. The number of mice used was also too small to be significant.

The 19 mice of the BC strain that had intravaginal pellets of estrone fixed in the upper vagina survived, on the average, 269 days, with a range of 161 to 356 days. Pellets were found in none of these mice at necropsy, although 2 mice had pituitary adenomas, which indicated that the pellets had remained in place for some time. The uterine and vaginal tissues usually were atrophic, in keeping with the age of the mice, which averaged 615 days. One mouse had a large spindle-celled sarcoma enlarging and invading the entire cervix and extending down into the vagina.

The 112 mice bearing pellets of collodion or of cholesterol, or retained as untreated animals, survived as well as did mice of any of the hormone-treated groups (TABLE 3). One vaginal cancer appeared in one untreated mouse of the BC strain. This tumor was small, only 3 × 4 mm. in diameter, but was evident at necropsy as a small ulcerated lesion. It was composed of highly anaplastic epidermoid cells and had extended through the vaginal wall to involve the perirectal tissues. The mouse in which this tumor appeared was 401 days old, had had 5 pregnancies, and had given birth to 4 litters that were recorded prior to being set aside to be included in this experiment. The appearance of these tumors must be extremely rare in untreated mice. They have not been found among the large number of untreated mice examined routinely at necropsy. One of the ancestral stocks from which the BC strain was derived did show some spontaneous vaginal and uterine cervical epidermoid carcinomas.[9] The one mouse with a vaginal carcinoma was the youngest mouse to come to necropsy in its particular group (TABLE 3).

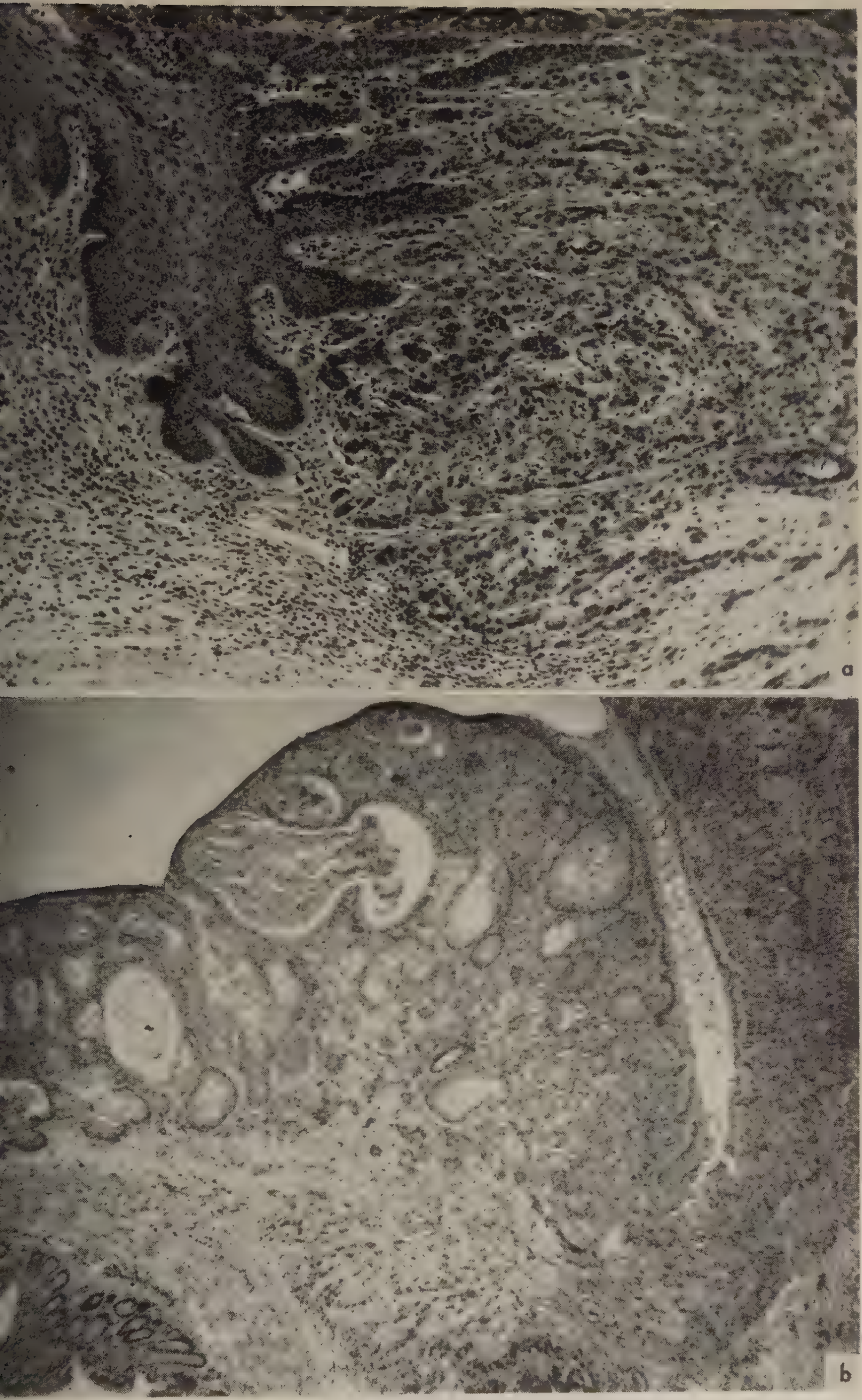

FIGURE 7. (a) Photomicrograph of an area of the tumor shown in FIGURE 6—a stage-4 esion (×120); (b) photomicrograph of the vaginal portion of the same tumor, showing the xtensive areas involved, and extension into the outer rectum at the lower left-hand corner. ×30).

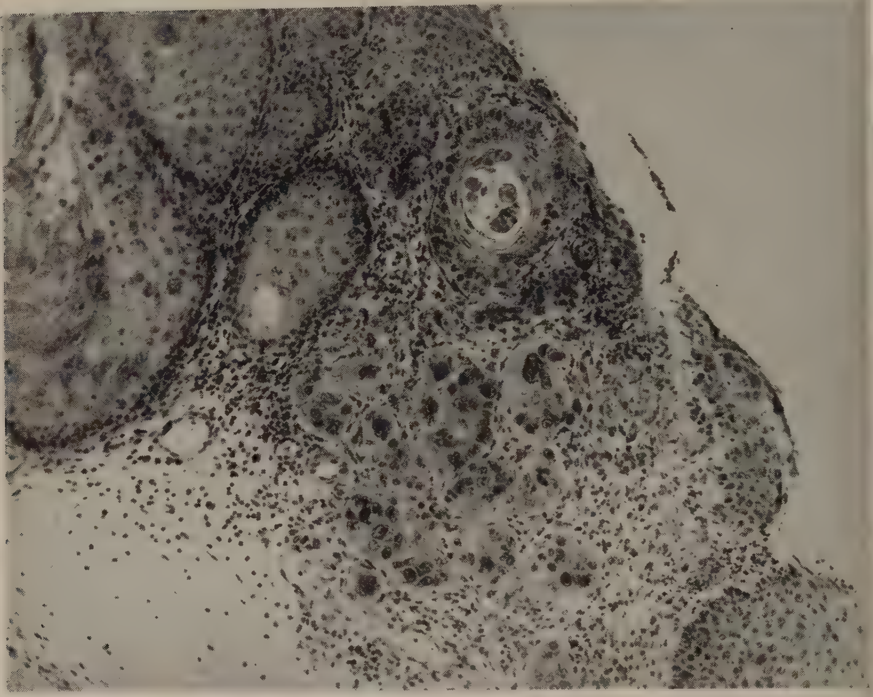

FIGURE 8. Photomicrograph of a small area of a peripheral part of a stage-4 carcinoma of a mouse that had an intravaginally placed pellet of stilbestrol-cholesterol for 256 days, beginning at 413 days of age. ×120.

None of the mice that had pellets of collodion implanted into the upper vagina had vaginal or cervical cancers, although they survived, on the average, 250 days. One mouse of the BC strain that survived 371 days after having a pellet of cholesterol implanted into the upper vagina had a small lesion that was rated 1−. The basement membrane was irregular but intact, and the cells were not too abnormal or unusual in appearance. This small area might not have been detected in an estrogen-treated animal. One mouse had an epidermoid lesion of the vulva at necropsy 128 days after the pellet had been implanted. Although this tumor was papillary, the base seemed to be invasive and it was probably malignant. Tumors of this type appear occasionally along the ventral body wall in mice of this strain.

Six of 13 mice of the BC strain that had pellets of stilbestrol-testosterone propionate placed in the upper vagina had epithelial lesions at necropsy (TABLE 2). These all appeared in mice that had retained their pellets until necropsy. The pellets were not present at necropsy in 6 mice; again, it was unknown how long they had remained in place. In all but 1 mouse the lesions were multiple. The large lesions tended to involve large areas of the vaginal wall and to spread in the mucosa rather than to penetrate it.

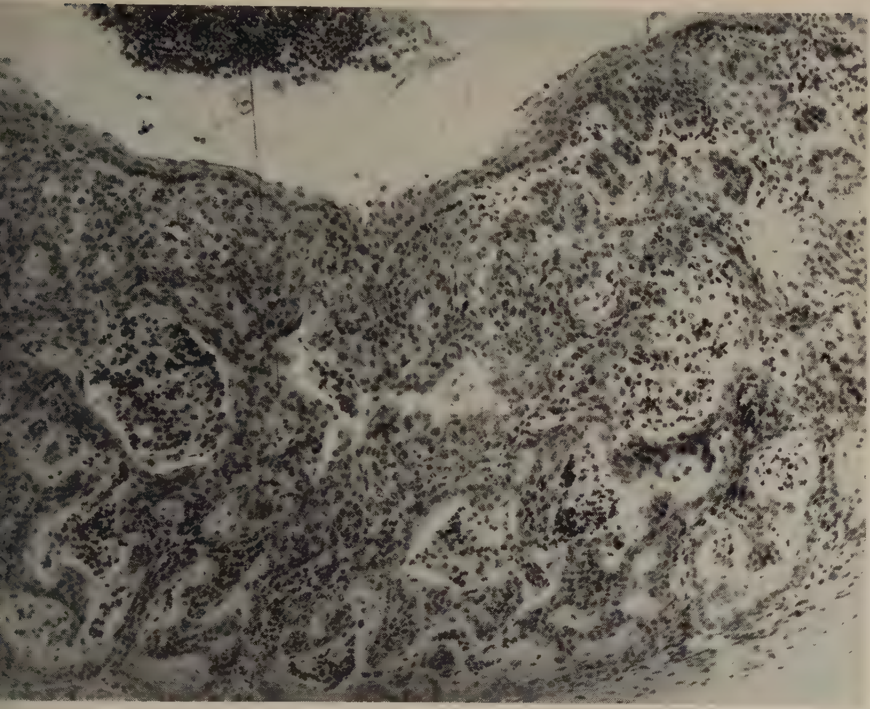

FIGURE 9. Photomicrograph of a section of a stage-4 carcinoma in the vaginal wall of a nouse that had a pellet of stilbestrol-cholesterol placed in the vagina for 274 days, beginning t 438 days of age. ×120.

The mice that had received pellets of methylcholanthrene or of progesterone ad no neoplastic uterine cervical or gvainal lesions (TABLE 2). Pellets were ot found at necropsy in any of these animals. One mouse that had received pellet of methylcholanthrene had a widely disseminated epidermoid carinoma, involving the diaphragm, pancreas, and stomach, that had apparently risen from the forestomach. This mouse had survived 360 days after the ellet had been implanted. Another mouse that survived 373 days had a apilloma of the lower lip.

Two mice had epidermoid carcinomas of the cervix and upper vagina after stradiol benzoate or stilbestrol had been injected subcutaneously at weekly ntervals for 311 and 266 days, respectively (TABLE 4). These tumors were istologically comparable to those described above. Seven of these mice had ubserosal uterine lesions of an adenocarcinomatous type described preiously.[8]

The application intravaginally of 10 mg. of the powdered base 3 times eekly for prolonged periods resulted in the appearance of vaginal epidermoid ancers in 10 of 35 mice and of hyperplastic lesions in 11 others (TABLE 5).

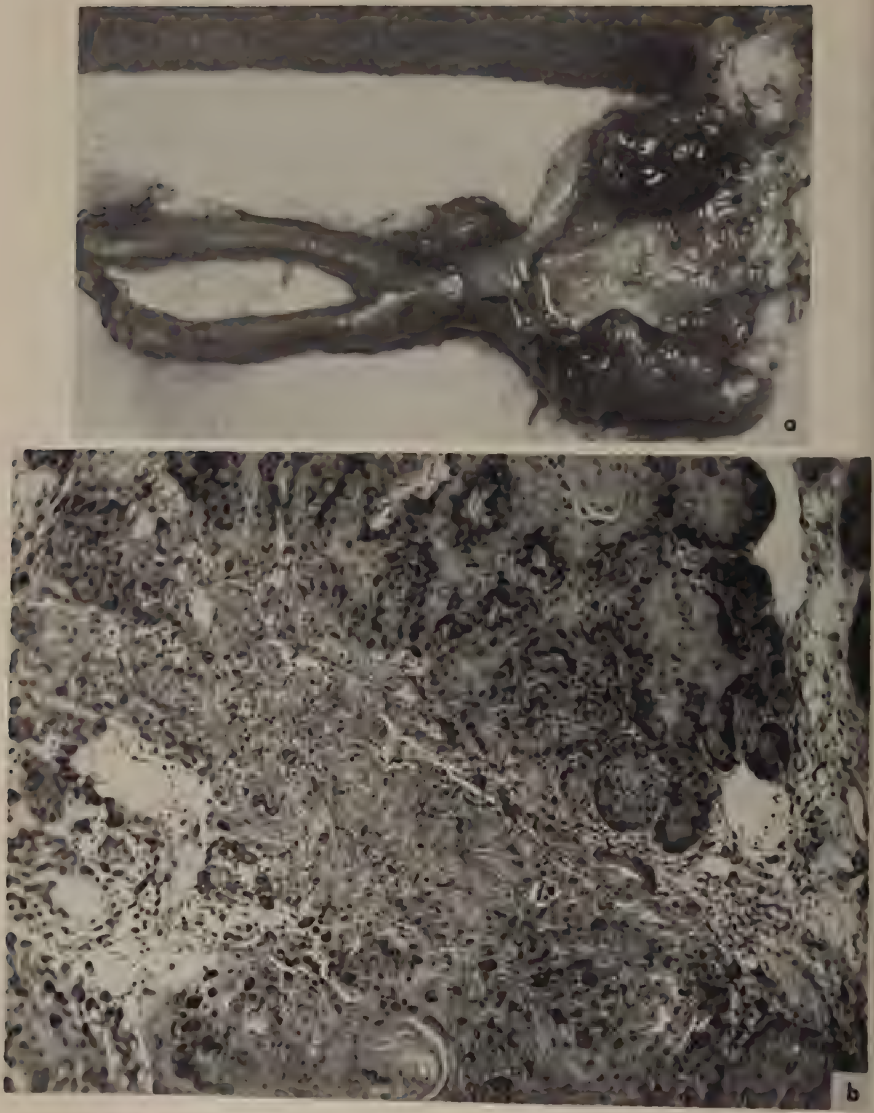

FIGURE 10. (a) Photograph of the opened vagina, uterus, and uterine cervix of a mouse that had received intravaginal application of [illegible] 3 times weekly for 524 days, beginning at 72 days of age. The tumor, a stage-4 lesion, was incised when the vagina was opened ventrally. (b) Photomicrograph of the border of the same carcinoma. ×120

These lesions were not unlike those that appear in mice given estrogens for prolonged periods (FIGURES 10–13). They occurred throughout the vagina and fornices and often were multiple when not so large as to involve most of the vagina. They appeared only after prolonged periods of treatment ranging

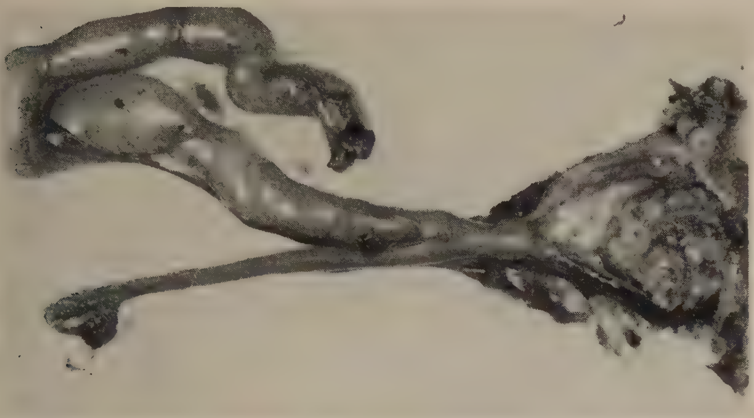

FIGURE 11. Photograph of the opened vagina, uterus, and uterine cervix of a mouse of he BC strain that had received intravaginal applications of base 3 times weekly for 526 days, eginning at 111 days of age. The tumor is a stage-2 lesion consisting of an epidermoid papiloma.

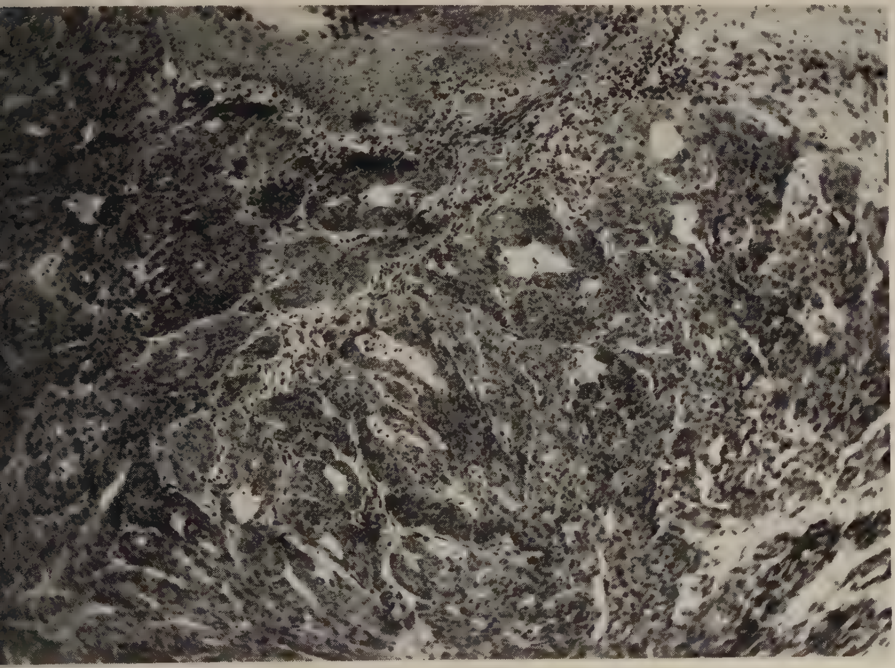

FIGURE 12. Photomicrograph of a carcinoma of the upper vagina of a BC-strain mouse hat had received base intravaginally 3 times weekly for 423 days, beginning at 126 days of ge. Some vaginal epithelium is located in the upper center. ×120.

from 426 to 631 days (TABLE 5B). The smaller lesions appeared after about the same period of exposure to the base as did the larger tumors.

The nontumorous areas of the vaginal mucosa or the vaginal mucosa of those mice that did not acquire neoplastic lesions were not unlike the tissue of untreated mice of comparable ages. The internal portion of the vagina was usually lined by a low stratified epithelium with a luminal layer of columnar mucus-containing cells. This type of epithelium occurs in most aged mice or in ovariectomized mice. Occasionally areas of cornified cells were interspersed among the mucus-containing cells. Nearer the vaginal orifice the vaginal epithelium was usually of the low stratified squamous type, although not lined by cornified cells or containing only scattered areas of cornified surface cells.

Mice that were older at the time the intravaginal applications of base were started showed fewer vaginal lesions. Only 2 of 21 mice, 17 of them over 300 days of age were first subjected to treatment, had small lesions at necropsy (TABLE 7). Ten mice survived less than 400 days after the injections were begun, and only 2 survived more than 500 days.

The intravaginal application of about 7.3 mg. of the preparation designated "mixture" 3 times weekly resulted in the appearance of only 1 vaginal cancer,

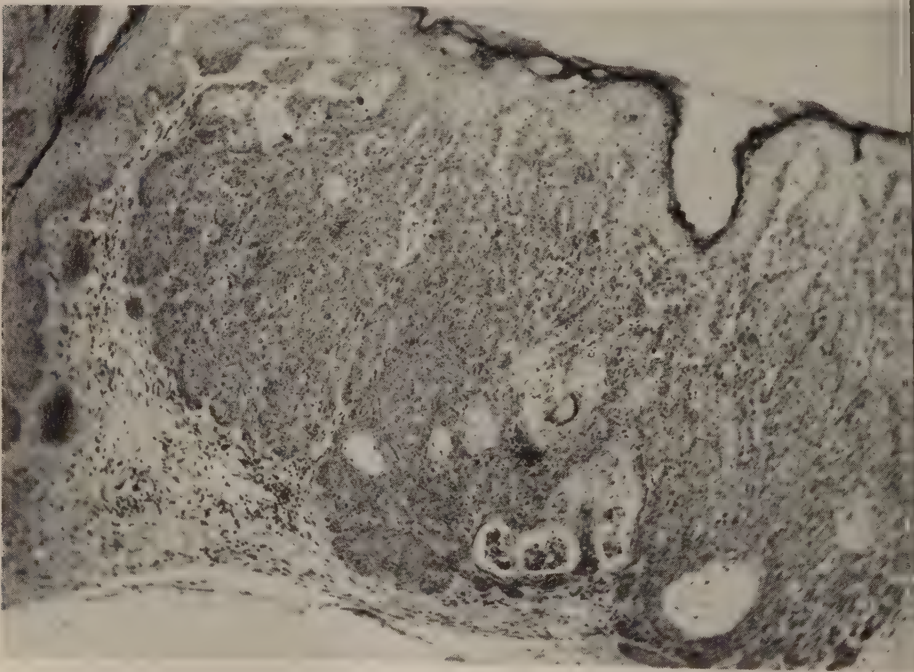

FIGURE 13. Photomicrograph of an area of an epidermoid lesion (stage 3) of the vagina in a CBA-strain mouse that had received intravaginal application of base 3 times weekly for 570 days, beginning at 132 days of age. This mouse also had 2 other small stage-4 lesions. ×120.

TABLE 7

(A) SUMMARY OF OBSERVATIONS ON UTERINE CERVICAL AND VAGINAL CANCERS AMONG MICE OF THE BC STRAIN GIVEN INTRAVAGINAL APPLICATION OF BASE* 3 TIMES WEEKLY BEGINNING AT AGES RANGING FROM 168 TO 469 DAYS (AVERAGE 327 DAYS)

	Total number	Age at death	
		Range	Mean
Mice with lesions	2	661–792	726
Mice without lesions	17	576–813	669
Total	19	576–813	657

(B) LESIONS AND AGE AT WHICH THEY WERE OBSERVED

Stage	1	2	3	4
Number	2			
Range (days)	412–479			
Average (days)	445			

* See TABLE 5

as determined by examination at necropsy. Most of the 37 mice in this group survived over 500 days. Since histological studies of the mice in this group are not complete, this report is only tentative.

Discussion

The high incidence of uterine cervical or, more frequently, vaginal epidermoid carcinomas among mice bearing intravaginal pellets of stilbestrol-cholesterol is considered to be of significance. Eight of 15 mice surviving, on the average, 260 days, had cancers, and 15 of 17 mice that had the hormone-containing pellets at necropsy had either cancers or infiltrative epithelial lesions (TABLE 1) The absorption of hormone from the pellets apparently was continuous over a prolonged period. Although levels of estrogen were attained that gave systemic responses, as determined by uterine and pituitary hypertrophy, the levels of hormone to which the adjacent tissues were exposed undoubtedly was relatively high. The topical application of hormones has been used as a means of determining their possible direct action on end organs[15, 16] Estrone and stilbestrol applied to the vagina are more effective in inducing vaginal cornification than when given systemically,[16] an observation that leads to the assumption that they act directly in eliciting vaginal growth and cornification. It seems reasonable to assume that they may act directly to incite vaginal or cervical cancers.

Two major objections might be raised to the above extrapolation:[17] (1) the prolonged delay between the onset of exposure to hormones and the appearance of cancers or epidermoid lesions does not permit such a nice temporal correlation of stimulation and response; and (2) the effect of the estrogens upon vaginal hydrogen ion concentration, the vaginal flora, or other changes that might be more directly associated with the carcinogenic response still make it possible that estrogens act indirectly. Nevertheless, these observations are probably the most convincing made thus far to the effect that estrogens are carcinogenic.

If the above assumption is correct, it seems rather useless, as far as relationship to cancer is concerned, to look for unique metabolites of hormones as the active carcinogenic agents. The potentiality for a neoplastic response exists in the end organ—the vagina—and direct exposure to estrogens provokes a response more rapidly than does systemically administered hormone. Quantitive differences in the responses of the vaginas of mice of different strains are in the vagina itself rather than in strain differences in capacities to inactivate or counteract the hormones.

The appearance of the vaginal cancers in animals that had ceased to show normal vaginal responses to the estrogens again was reaffirmed.[11, 12] The vaginal epithelium was not as thick or as uniformly cornified as it had been after less intensive hormone treatment during shorter periods of time. The vaginal mucosa seemed to be responding abnormally in other than the initiation of neoplasia. The possibility that the abnormal tissues may be producing a tumor-evoking agent must be considered. The similarity of the vaginal responses to the topical application of hormones and to systemic injections[17] indicates similar pretumoral changes.

The failure of placebos to incite vaginal cancers indicates some specificity of the response.

The large number of vaginal cancers in the mice that received the intravaginal application of the three chemicals, urea, adipic acid, and carboxymethylcellulose acids, indicates a lack of specificity of the carcinogenic response. The periods of application necessary for the appearance of cancer were prolonged. These observations, however, would indicate that prolonged exposure to even unsuspected chemicals might evoke cancer or be associated with its evocation. Substances used for prolonged periods or associated with intravaginal contraceptives probably should be checked carefully. The appearance of multiple cancers or tumors in rats, particularly in rats on a low protein diet given certain contraceptive preparations either orally or intravaginally, indicates the possible carcinogenic activities of some of these preparations.[18] At least one vaginal or cervical carcinoma appeared in rats so treated.

The only substance in the mixture of chemicals employed in the last part of this study that has been implicated in carcinogenesis is the carboxymethylcellulose.[19] The subcutaneous injection of a 2 per cent suspension of this substance into rats for 73 weeks resulted in the appearance of sarcomas in 43 per cent of the recipients. Experiments are not advanced sufficiently in our laboratory to permit a statement other than that tumors occurred when the 3 substances were given concurrently.

The cancers and invasive epithelial lesions in the mice that received the chemicals were comparable to those that appeared in estrogen-treated mice or in the 1 untreated control mouse. Because they appeared in mice after 500 days of treatment, on the average, they usually appeared in animals with atrophic ovaries and uteri. The nontumorous vaginas, however, did not differ from those of untreated animals of comparable ages. Likewise, the non-tumorous areas of those mice with cancers involving only a portion of the vagina were histologically normal in appearance. This would indicate that the substances, as given, were not generally toxic to the tissues, and that localized foci gave origin to the neoplasia.

Summary

(1) The intravaginal instillation 3 times weekly of 1 to 4 μg. of stilbestrol dissolved in sesame oil resulted in the appearance of epidermoid carcinomas in the vagina or vaginal fornices of 3 of 21 mice so treated, and of small invasive epithelial lesions in 5 mice.

(2) Epidermoid carcinomas appeared in the vaginas or vaginal fornices of 8 of 40 mice of the BC strain in which pellets of stilbestrol-cholesterol were fixed in the upper vagina. Smaller lesions were found in 12 other mice. The period of exposure to estrogen averaged 260 days. Vaginal or cervical carcinoma did not appear among 14 mice of other strains that were similarly treated. They did not tolerate treatment as long as did mice of the BC strain.

(3) One of 112 control mice, either untreated or bearing intravaginal pellets of collodion or cholesterol, had a vaginal cancer at 401 days of age.

(4) Six epithelial lesions of the vagina or cervix appeared among 13 mice bearing intravaginal pellets of stilbestrol-cholesterol-testosterone propionate for an average period of 253 days.

(5) Invasive epithelial lesions or cancers appeared among 15 of 37 mice of the BC strain given either estradiol benzoate (16.6 μg. weekly) or stilbestrol (either as pellets or in oily solution).

(6) Twenty-one of 35 mice receiving 3 times weekly an average of 10 mg. of a powder containing urea, adipic acid, and carboxymethylcellulose acid had invasive epithelial lesions of the upper or central vagina. Ten mice had lesions considered to be cancers. Periods of treatment in excess of 500 days were required.

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HYDATIDIFORM MOLE AND CHORIOCARCINOMA

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The two diseases discussed in this paper, only one of which is an authentic neoplasm, are sojourners and not true uterine natives. Hydatidiform mole, of course, is a placenta, seriously deformed structurally and occasionally aggressive in behavior, while choriocarcinoma is a highly malignant neoplasm composed of trophoblastic cells, usually of gestational origin in the female, but not uncommonly of teratoid origin in the female and almost always so in the male. Since this monograph is oriented toward the uterus, I shall discuss only the gestational form of choriocarcinoma involving the uterus.

Hydatidiform mole is, in all probability, a malformation of the placenta, fundamentally characterized by complete or almost complete failure of vascularization of the villi, followed by severe swelling and the formation of the typical watery, grapelike "hydatids." The trophoblast covering the swollen villi is extremely variable in appearance and behavior, not only from case to case, but from villus to villus. The grossly defective vascular structure, of course, is incompatible with continued existence and growth of the fetus. As a consequence, in cases of true fully developed hydatidiform mole, the fetus is either absent or severely stunted. Exceptionally, one placenta in a twin pregnancy becomes molar; the simultaneous presence of a normal fetus and an obvious mole in the same uterus has been known to confuse the unwary.

In the usual course of events, hydatidiform mole evacuates itself or is evacuated with little or no deleterious effect on the uterus. In perhaps 1 per cent of cases of hydatidiform mole, the villi are endowed with sufficient growth activity to invade the myometrium or even the parametrium, but rarely, if ever, produce distant metastases. To this form, a variety of terms, including chorioadenoma destruens, invasive mole, and (unfortunately) malignant mole have been applied. Estimates of the frequency of invasive mole may well be low since, for all practical purposes, the diagnosis can be confirmed only by hysterectomy. There is reason to believe that, on occasion, invasive mole may be a self-limiting disease, as indicated by the finding of degenerating molar villi within the myometrium some time after passage or evacuation of a typical hydatidiform mole.

Much of the clinical apprehension associated with the diagnosis of hydatidiform mole is, of course, due to the occasional subsequent development of choriocarcinoma. The risk is of the order of less than 2 per cent, based on the subsequent development of choriocarcinoma in four series of hydatidiform moles, carefully studied and carefully followed (Hertig and Sheldon, 1947; Hunt *et al.*, 1953; Novak and Seah, 1954; Grady, unpublished). On the other hand, choriocarcinoma may follow any type of pregnancy, molar, normal full term, or spontaneous abortion. About 75 per cent of gestational choriocarcinoma follows molar or full-term pregnancies.

Choriocarcinoma, commonly called chorionepithelioma, was known under a variety of descriptive terms before Marchand (1895) clearly established the trophoblastic nature of the neoplastic cells. John H. Teacher, of Glasgow, Scotland, was one of the early supporters (1903) of this view in the English literature (incidentally, Teacher's name is perpetuated not only by pathologists, but by several generations appreciative of a nondairy form of "Highland Cream").

Choriocarcinoma has fascinated pathologists and clinicians far beyond the frequency of its appearance. In Risel's review (1907), no less than 479 references appear in the bibliography, with the vast majority appearing during a 10-year period. There seems little doubt that there is considerable variation in frequency in different geographical areas, with high frequencies in the Orient and low in the United States and Western Europe. Recently, L. Iverson (personal communication) briefly reported observations on 104 cases of uterine choriocarcinoma collected in southeast Asia, a figure far greater than any series in Western Europe or the United States, including the material at Novak's laboratory in Baltimore, or the Armed Forces Institute of Pathology in Washington, D. C.

Classically, choriocarcinoma is a rapidly growing invasive tumor, particularly prone to spontaneous hemorrhagic necrosis. Like its normal counterpart, it has an avidity for blood vessels, which it erodes and into which it grows. In short order, metastases appear in the lungs, liver, brain and, on occasion, almost every organ in the body, although lymph nodes are rarely invaded. As a result, the vast majority of patients with this disease die with and because of it within 1 year. In spite of such a usual devastating course, a cure rate of perhaps 10 to 15 per cent may be expected (17.5 per cent 1-year survivals in the 74 cases studied by Novak and Seah, 1954).

Histologically, the tumor reproduces the characteristic appearance of the young trophoblast, both cytotrophoblast and syncytiotrophoblast, with no more than the usual distortion expected in neoplastic cells. Both cell types are easily recognizable, but there is no villous core supporting them, and their normal spatial relationships frequently are sadly jumbled. As is well known, chorionic gonadotrophins are produced in abundance and form the basis for hormonal diagnosis.

Exceptionally, choriocarcinoma apparently undergoes spontaneous regression, at least in the primary site and possibly to a lesser degree in metastatic locations. Many of these cases are difficult to evaluate, but I have no doubt that this phenomenon does occur. Whether the mechanism is related to spontaneous hemorrhagic necrosis (a sort of neoplastic suicide) or whether this is a response to unfavorable hormonal environment, perhaps a "conditioned" neoplasm in the sense of Jacob Furth is conjectural.

To the best of my knowledge, neither hydatidiform mole nor choriocarcinoma has been recognized in any except the human species. A few reports of tumors similar to choriocarcinoma in various nonhuman species have not stood up under critical scrutiny. This is disappointing, since experimental approaches to the problem of trophoblastic tumors have been sadly hampered as a result.

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UTERINE SARCOMAS: HISTOGENESIS AND TAXONOMY

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Before proposing a system of classification, I should like to consider the histogenetic aspects of uterine sarcomas. In most cases, histogenesis is straightforward once the neoplasm has been classified correctly. For example, leiomyosarcoma, the most frequently encountered form, is derived from adult smooth muscle. Tumors of blood-vessel origin are derived from pre-existing adult blood vessels, which are ubiquitous in the uterus as in any other viscus. The few instances of lymphosarcoma or reticulum cell sarcoma that arise in the uterus either *de novo* or as part of multicentric tumorigenesis are derived from lymphoid or reticuloendothelial cells, which are likewise ubiquitous. The only group of uterine sarcomas that poses a histogenetic problem is the group of so-called "mixed" or "mixed mesenchymal" or "mixed mesodermal" tumors. This group is of particular importance because, historically, there has been much debate concerning its nature, particularly with respect to explanations for the diversity of cellular elements it may contain and with respect to the problem of the relationship of malformation to tumor.

Many of the theories held in the past have been discarded as either not probable or not provable. For example, J. F. Cohnheim's theory of displaced rests is not given much credence today. M. Wilms' theory, which was a slightly more sophisticated version of the rest theory, implied an invisible rest, that is, a group of cells indistinguishable from normally matured cells, but lacking the capacity for coordinated response to normal stimuli for growth and differentiation. Wilms explained the presence of cartilage and bone in mixed tumors on the basis of inclusion of structures from the sclerotome into the caudal portion of the growing Wolffian duct. J. E. Sweet applied Wilms' ideas to Müllerian duct derivatives as well. It is not clear from reading either Wilms or Sweet whether they thought their theories were a further definition of Schwalbe's concept of a "dysontogenetic field" or as something opposed to it. Robert Meyer advanced theories of displacement still further by postulating "illegal cell connections" between Wolffian and Müllerian systems as the origin of mixed tumors. Some reinforcement of Meyer's theory can be found in the observation of Gruenwald,[1] who demonstrated that the growing caudal end of the Müllerian duct is in contact with and surrounded by the same basement membrane as the growing end of the Wolffian duct. Possibly, Meyer's illicit cell connections may not prove to be as illegal as the embryologic knowledge of his day permitted him to think.

Theories based on rests are no longer in vogue. However, occasionally the presence of a displaced rest is noted. In only one case have I ever seen anything in a tumor that remotely might be considered a rest. This was in a case of sarcoma botryoides of the cervix of a 21-year-old woman reported by Edgcomb and myself.[2] In the middle of this poorly differentiated neoplasm with many atypical, bizarre cells there lay a structure composed of three plates of well-differentiated cartilage surrounded by a membrane suggestive of perichondrium. Whether this truly was a rest that had been there before the tumor developed and, if so, whether it had anything to do with the development of the tumor, I prefer not to say.

Contemporary with the above theories were ideas weighted more in terms of the dynamics of cell growth. J. Pfannenstiel's concept that the varied components of these tumors arose on the basis of metaplasia antedates Wilms' publications by several years. I should interpret Schwalbe's idea of a "dysontogenetic field" as an effort to reconcile these divergent emphases on the mechanism of tumor formation. E. Albrecht is in this tradition; he considered tumor formation to be a repetition of organ-forming processes, representing organoid formation in excess on the basis of an embryonic or a postembryonic error in development, never a primary error or malformation of a cell. He recognized the importance of postnatal pathological processes in tumor formation.

G. W. de P. Nicholson is the intellectual heir to Albrecht's theories. In addition to Albrecht's ideas of anomalous blending, he also described anomalies of bulk, position, and differentiation. Although it must be difficult to be certain which type of anomaly is dominant in any given tumor, the only real criticism I have of Nicholson's ideas is that his concept of "acquired tissue malformations" can be made so broad as to be nondefining. Nicholson ascribed the rich variety of cellular elements to a process of "dedifferentiation and rejuvenescence," a process that assumes particular significance in the ight of subsequent discoveries about the lack of a sharp division between stromal and epithelial cell forms. Parenthetically, Nicholson's cogent powers of ratiocination were applied to mixed uterine sarcoma in an infrequently consulted case report.[3] I commend it to your attention as a landmark in a iterature consisting chiefly of poorly documented, poorly illustrated, even naccurate case reports.

Most recently, Willis[4] has expressed himself with characteristic emphasis: "Many workers have followed Wilms in supposing the uterine mixed tumors to arise from developmentally misplaced tissues. This view should be discarded; it is discredited by the researches of experimental embryologists, and t is as unnecessary as it is improbable. The supposition that undifferentiated rests of heterotopic tissues may remain in the endometrium for the whole of a woman's reproductive life, surviving multiple pregnancies, and eventually producing a mixed tumor at the age of 50, 60, or 70, is absurd." I concur nsofar as postmenopausal women are concerned. Whether or not this should apply to histogenetically similar tumors such as the "juvenile" sarcoma botryoides remains to be seen.

In developing theories of histogenesis, one must keep in mind that the endometrial stroma and its congeners distributed throughout the genital tract, although mature, are the least highly specialized adult derivatives of the mesenchyme of the urogenital ridge. The distribution of the tissue beneath he peritoneal surfaces of the uterus and other pelvic structures (forming the subcoelomic mesenchyme) is the basis for the occurrence of histologically and histogenetically similar tumors arising outside the *cavum uteri* and its accessory passages.

Gruenwald[5] has demonstrated that in the human embryo the mesenchyme around the müllerian duct is derived from the coelom even as duct epithelium itself. As the duct develops, there are continuing contributions from the surrounding mesenchyme to the ductal epithelium and vice versa. It might be more accurate to speak of a müllerian apparatus in which the mesenchyme and epithelium are siblings with some capacity for morphologic intermutability, or lability. However, to some extent this property must be lost as cell maturation and specialization progress, for we do not see epithelium regenerating from stroma or vice versa in endometria examined after curettage or during the puerperium. Even if the glands and stroma were not related ontogenetically, one can conceive of tumorigenic stimuli that are either selective and specific or the converse. Tissues have only a limited number of responses to stimuli; neoplastic growth is merely one. The number of possible stimuli is almost infinite.

Finally, mixed tumors are not reported in the fallopian tube, the unfused portion of the müllerian apparatus, whereas a surprising number arise either in the anterior or posterior midline of the uterus or at the junction of the upper third with the lower two thirds of the vagina. Whether there is increased cellular lability at lines of fusion or whether there is an anomaly of blending at these sites remains an unanswered question.

The taxonomic study of tumors is largely the province of surgical pathology. Insofar as uterine sarcomas are concerned, I need stress only the importance of studying anatomically well-oriented sections, of studying multiple sections, of realizing the value of special stains in demonstrating specific features of the cells and ground substance, and of noting the distribution and composition of metastases. Post-mortem studies furnish the necessary information regarding distant metastases, the natural history of the disease, and the mechanism of death. As a pathologist, I think it is important to have a clear idea of the morphologic distinctions between groups of related tumors so that their behavioral differences can be set in a meaningful context. I am aware that it is currently fashionable in some circles to dismiss such studies as "mere morphology"; I attribute much of the confusion in the literature regarding uterine sarcomas to an imperfect grasp of the morphologic aspects of the problem. That many of those who disparage mere morphology are those who could profit from it the most is a paradox that fails to stir my risibilities.

Uterine sarcomas are divided into six major classes (TABLE 1). Of these, leiomyosarcoma is the most frequently encountered but, histologically, it is the least varied. The family of mesenchymal sarcomas is next most frequent and is histologically the most complex. Tumors of blood vessel origin are extremely rare, but they present a few interesting relationships. Lymphomas are included for the sake of completeness. I know of no case of metastatic sarcoma (unless one includes leukemic infiltration). Unclassified sarcomas are always a problem.

TABLE 1

A CLASSIFICATION OF UTERINE SARCOMAS

(1) Leiomyosarcoma
  - Arising in a leiomyoma
  - Arising diffusely in the uterine wall

(2) Mesenchymal Sarcoma
  - Pure—homologous
    - Endometrial stromal sarcoma
    - Stromatous endometriosis (endolymphatic stromal myosis)*
    - Sarcoma botryoides (without heterologous elements)
  - Pure—heterologous
    - Rhabdomyosarcoma
    - Chondrosarcoma
    - Osteosarcoma
    - Liposarcoma (not reported)
  - Mixed—Homologous
    - Carcinosarcoma
      - Adenocarcinoma plus stromal sarcoma
      - Adenoacanthoma plus stromal sarcoma
      - Squamous carcinoma plus stromal sarcoma
      - Sarcoma botryoides plus neoplastic epithelium and without heterologous elements
  - Mixed—Heterologous
    - Carcinosarcoma (as above, with one or more heterologous elements)
    - Mixed mesenchymal sarcoma
      - Stromal sarcoma plus one or more heterologous elements
      - Two or more heterologous elements without mesenchymal "myxomatous" stromal sarcoma
    - Sarcoma botryoides (with heterologous elements)

Note: Cartilage and striated muscle are the most frequent heterologous elements. Bone is occasionally seen. Heterologous lipoblastic elements are difficult to evaluate.

(3) Blood Vessel Sarcomas†
  - Hemangiosarcoma (hemangioendothelioma)
  - Hemangiopericytoma*

(4) Lymphomas
  - Reticulum cell sarcoma
  - Lymphosarcoma
  - Leukemic infiltration

(5) Unclassified sarcoma

(6) Metastatic sarcoma (not reported)

* These tumors are not uniformly malignant.
† Lymphangiosarcoma has also been reported.

Leiomyosarcoma, statistically the most frequently encountered uterine sarcoma, comprises from 50 to 75 per cent of various large series. It may arise in a pre-existing leiomyoma or, less frequently, may arise diffusely in the wall of a nonmyomatous uterus. In some instances it may be difficult to ascertain from the gross appearance whether it arose in a leiomyoma or not. In a recent article, Corscaden and Singh[6] have distinguished between "lethal" and innocuous cases. They point out that, when the "sarcoma" is defined as cellular atypicalities confined to a leiomyoma without evidence of invasion of the surrounding myometrium, the tumor is harmless. Usually such cases are discovered by the pathologist upon histological examination of one or more leiomyomata in a uterus removed without clinical or even gross pathological suspicion of malignancy. This is a form of "pathologist's cancer." The opposite picture is presented by leiomyosarcomas that are grossly and diffusely invasive. These sarcomas tend to appear clinically as large multinodular masses, often with a history of recent rapid growth. At exploration they are often adherent to adjacent structures or have infiltrated widely, and casual gross inspection leaves little doubt as to their malignant nature. In Corscaden's series, all these patients died of their disease in short order.

One is tempted to ask if the "sarcoma" consisting of atypical cells still confined by the pseudocapsule of a leiomyoma is analogous to carcinoma *in situ*, that is, would it have invaded sooner or later if it had been left to its own devices? I hesitate to give a quick and ready answer to that query. Certainly, not all leiomyosarcomas grow and infiltrate at the same rate. Very rarely one encounters a case in which leiomyomata have been removed 10, 15, or 20 years before a large pelvic recurrence develops and, on closer examination of the original tissue or on further sectioning, one may find areas of cellular atypism that, in retrospect, are reclassified as sarcomatous. The statement of this problem by so distinguished an observer as Corscaden points out our need for careful study of these cases with detailed follow-up information. I hope that information gained from prospective studies will be of more value than that gained from retrospective studies.

It is important to distinguish clearly from sarcomas of muscular origin the rather infrequent tumors of connective tissue origin that occur in the uterus. The example I have in mind is the lymphangiocystic fibroma. The only two examples of this lesion I have seen were originally misdiagnosed, one as a leiomyoma, the other, because of its cellularity, as a leiomyosarcoma. The best description in English of this tumor is that by Plaut,[7] who described the fourteenth case to be recorded. So far as we know, the lymphangiocystic fibroma is benign in its behavior.

The next major group of sarcomas embraces a large group of neoplasms that arise in the endometrium and are composed either of neoplastic stromal elements or an admixture of stromal and epithlial elements. The terms we have applied to the individual members of this family are all recognizable either as "cell specific" or as "group specific," that is, the terms chondrosarcoma and sarcoma botryoides and carcinosarcoma are not innovations. We have attempted to classify these tumors in such a way as to emphasize their histogenetic and histologic interrelationships, subdividing the categories in such a manner that they can be used to relate the gross and microscopic features of a tumor to its subsequent behavior.

The general term we have adopted for this group is mesenchymal sarcoma; essentially, it is synonymous with the terms "mixed mesodermal tumor," "mixed Müllerian mesenchymal sarcoma," and "combined mesenchymal sarcoma and carcinoma" insofar as these terms apply to the microscopic features. The term "mesenchymal sarcoma" has the advantage of brevity. We prefer the term "mesenchymal" to "mesodermal" because it describes accurately the cellular element common to this family, whereas the entire uterus is of mesodermal origin. The term "mesenchymal sarcoma" unfortunately does not indicate that a considerable proportion of this group of tumors consists of cases in which there is a carcinomatous element; however, it is the presence of the mesenchymal or stromal component that gives this group of tumors its distinctive microscopic appearance and distinguishes it from the garden varieties of uterine carcinoma.

The major subdivisions of the group are based upon the ideas of Zenker,[8] who in 1864 classified uterine sarcomas in respect to whether they were pure, that is, composed of tissue of one cell type, or mixed, that is, more than one cell type, or whether they were composed of homologous elements, that is, direct derivatives of cells normally found in the uterus, or heterologous elements, that is, containing cells or tissues not normally indigenous to that organ. It can be seen that this concept necessitates placing tumors of certain types into more than one major subgroup. For example, a carcinosarcoma may be either homologous and contain a mixture of adenocarcinomatous elements indistinguishable from ordinary endometrial adenocarcinoma and neoplastic stromal elements indistinguishable from pure stromal sarcoma, or it may be heterologous and contain such diverse elements as skeletal muscle, cartilage, and bone. Whether or not such fine points in taxonomy are justified in terms of the biological behavior of these tumors remains to be seen. In any event, the proposed classification enables one to place a tumor with some degree of certainty into its position in the scheme of things and affords one a visual image that corresponds roughly with what one might expect to see.

Likewise, sarcoma botryoides can be found in three places in the classification. We have elected to retain this term because it connotes a distinctive gross appearance, a characteristic age distribution, and a topographic distribution, as well as other features that suggest that it may be pathogenetically different from the other varieties of tumor in the family. It is doubtful whether the ability of the pathologist to demonstrate heterologous elements or the rare presence of apparently neoplastic glands in botryoid sarcomas is of any real significance; however, if functional differences can be correlated at all with structural differences, this classification permits such analysis to be made.

I think we have passed beyond the era in which it is sufficient to classify uterine sarcomas as spindle cell, round cell, mixed cell, and giant cell. It is only fair to point out at this time that our vocabulary differs slightly from that employed with great skill by Symmonds and Dockerty,[9] who speak of fibromyxosarcoma (equivalent to our endometrial stromal sarcoma), carcinosarcoma (which they limit to those composed of homologous elements only), and mesodermal mixed tumors (which includes both carcinosarcomas with heterologous elements and what we term mixed mesenchymal sarcoma). This is merely a difference in vocabulary and does not reflect any disagreement in our conception regarding the origin and behavior of the tumors. The classification of Symmonds and Dockerty is much less elaborate than ours and probably much easier to use.

The common denominator to this entire family of mesenchymal sarcomas is the presence of a variable quantity of what we call endometrial stromal sarcoma. This is equivalent to Alznauer's[10] "undifferentiated cellular sarcoma,"

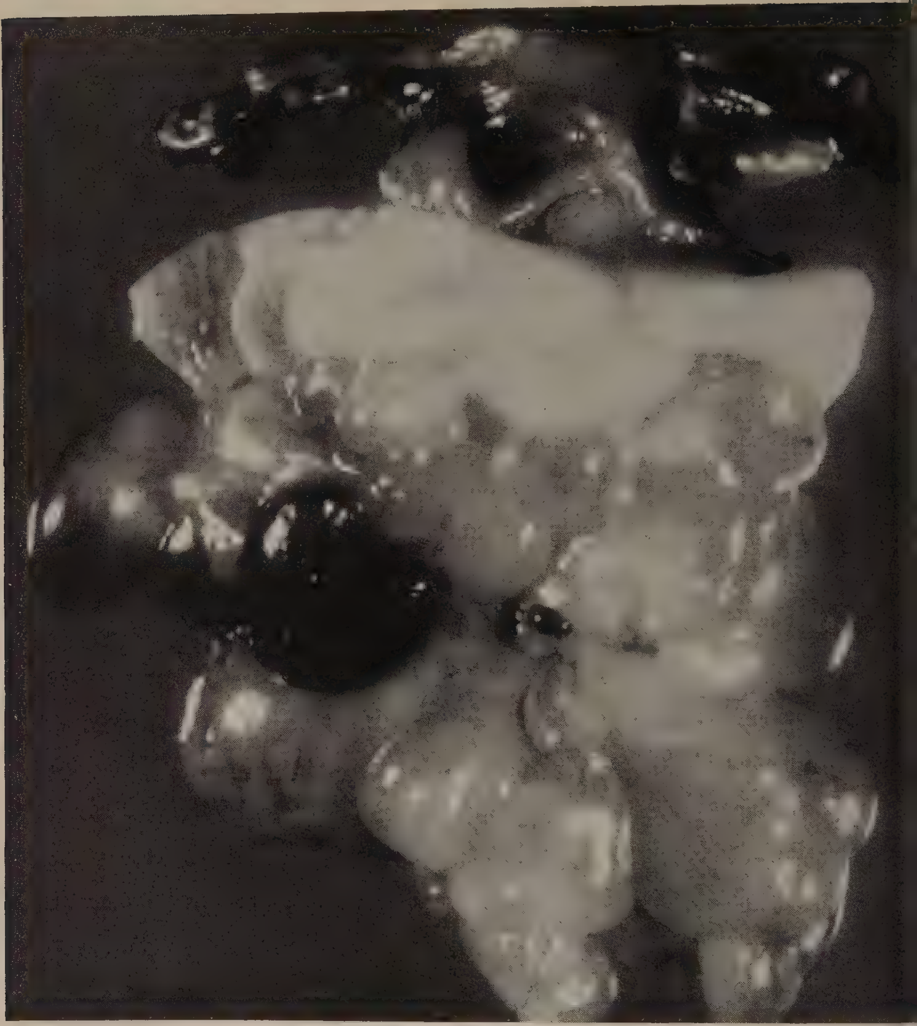

Figure 1. Sarcoma botryoides vaginae puellae. A hysterocolpectomy was done on an 11-month-old girl. The specimen shows the vaginal canal filled with large grapelike edematous polypoid structures. Courtesy of the late Sophie Spitz

Symmonds and Dockerty's "fibromyxosarcoma," and Sternberg, Clark, and Smith's[11] terms "endometrial sarcoma" and "unclassified sarcoma." It can be described best as a neoplastic proliferation of stromal cells of varying cellular densities, ranging from the loose, myxomatous appearance found in the central portion of the grapes of a botryoid sarcoma to a densely packed aggregation of small round cells exhibiting the nuclear stigmata of neoplasia and no specific cytoplasmic features of specialization. In looser areas one

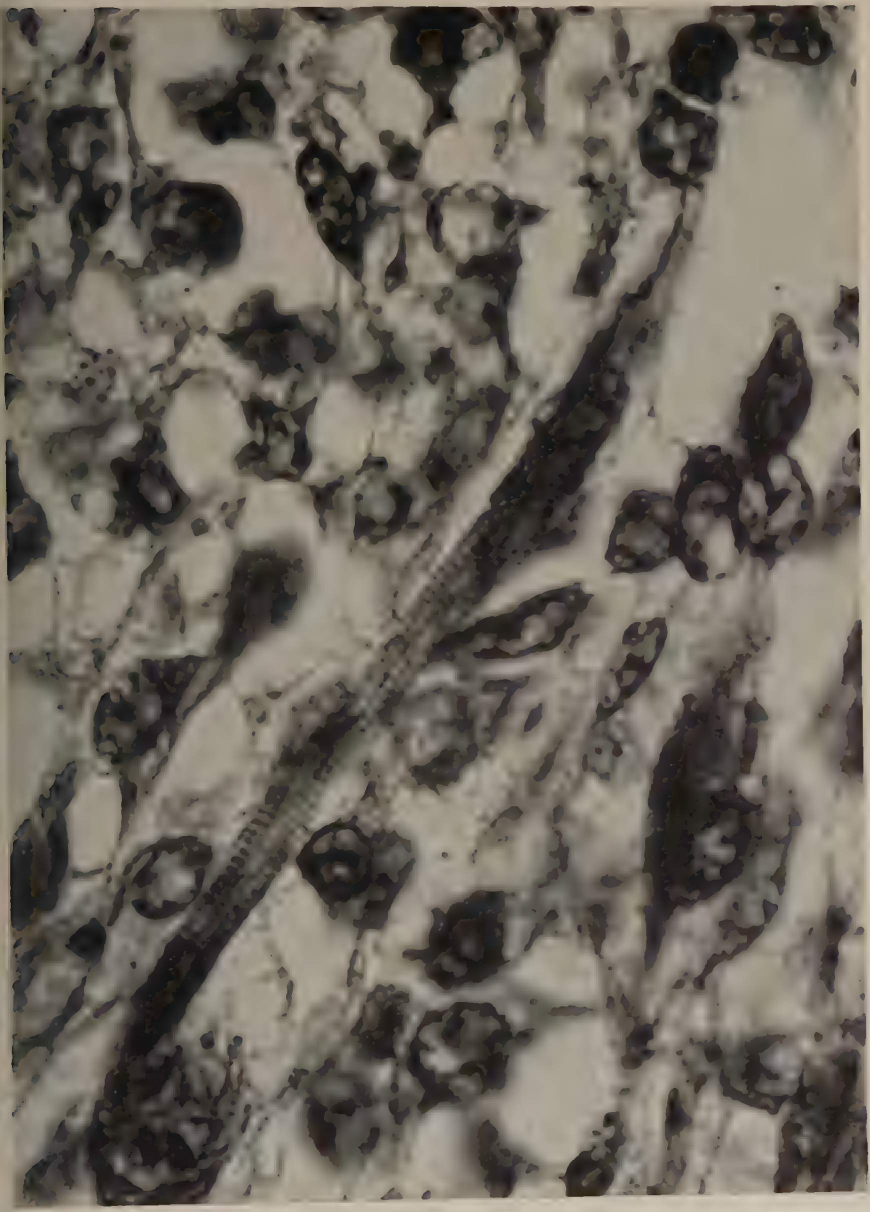

FIGURE 2. Field from a case of carcinosarcoma of the endometrium, showing an elongated cell with two separated nuclei, the intervening cytoplasm being rich with transverse striations. Elsewhere in the tumor there were numerous neoplastic glands. The cells surrounding the "rhabdomyoblast" are the cells of mesenchymal or stromal sarcoma. ×2000. Armed Forces Institute of Pathology Neg. No. 292563-1902

Table 2

Diagnoses in the Sloane-Delafield Series (Mesenchymal Sarcomas Only)

Endometrial stromal sarcoma		
Stromatous endometriosis		
Rhabdomyosarcoma		
Carcinosarcoma		1
Homologous	8	
Heterologous	8	
Mixed mesenchymal sarcoma		
Sarcoma botryoides vaginae puellae		
Total		2

Note: This enumeration does not include leiomyosarcomas, 1 case of hemangiosarcoma, reticulum cell sarcoma, and 1 lymphosarcoma.

sees a mixture of unipolar, bipolar, and multipolar (stellate) cells resembling the mesenchyme of the embryo. In more cellular areas the bipolar cells tend to become elongated, forming ill-defined interlacing fascicles; however, they do not deposit collagen (nor collagenize ground substance) and are not fibroblasts, and we prefer to avoid designating them as areas of fibrosarcoma or fibrosarcomalike growth on that basis. The unipolar and stellate cells, when condensed into cellular aggregates, form no distinctive pattern; they are seen as a mass of nuclei surrounded by rather scanty, ill-defined cytoplasm with indistinct cell boundaries. Stains for reticulum reveal an inconstant arrangement of delicate argyrophilic intercellular fibrils that often seem to be applied to almost every cell in cellular areas, but are extremely sparse in edematous areas. Collagen stains are likewise not revealing; at most, one sees sporadic collagen fibers that may or may not represent condensed reticulum and that may or may not represent pre-existing stroma. In myxomatous areas the ground substance is often metachromatic to toluidine blue. Traces of acid mycopolysaccharide can be demonstrated by patchy areas of faintly positive reactions with Schiff's periodic acid-leukofuchsin reagent.

The nosologic distribution of cases in the Sloane-Delafield series is shown in TABLE 2, and in the Armed Forces Institute of Pathology (AFIP) series in TABLE 3.

Of the 90 cases in the combined series, 47 are classified as carcinosarcoma (52 per cent), 27 fall into the group of pure homologous tumors, endometrial stromal sarcoma, and stromatous endometriosis (30 per cent), and 8 cases are classified as mixed mesenchymal sarcoma (9 per cent). There are also 8 cases of sarcoma botryoides (9 per cent) and 1 case each of chondrosarcoma and rhabdomyosarcoma. The question of "purity" in heterologous tumors of mesenchymal derivatives is usually solved on an arbitrary semiquantitative basis. For example, if a tumor is composed almost entirely of cells that have specialized as skeletal muscle elements (with or without demonstrable transverse striations) and there are no conspicuous patches of loose myxomatous stromal sarcoma or cellular stromal sarcoma, it might be classified as a rhabdomyosarcoma. Conversely, if there is conspicuous stromal sarcoma admixed

TABLE 3

DIAGNOSES IN THE ARMED FORCES INSTITUTE OF PATHOLOGY SERIES (MESENCHYMAL SARCOMAS ONLY)

Endometrial stromal sarcoma		14
Stromatous endometriosis		6
Chondrosarcoma		1
Carcinosarcoma		31
Homologous	8	
Heterologous	23	
Mixed mesenchymal sarcoma		5
Sarcoma botryoides		7
Total		64

Note: This enumeration includes the 2 cases of endometrial stromal sarcoma reported by Ober and Jason[16] and the 5 cases of sarcoma botryoides reported by Ober and Edgcomb.[2] It does not include the 2 cases of rhabdomyosarcoma of the vulva and vagina reported by Ober, Palmer, and Glassy.[44]

with extensive areas of specialized neoplastic tissue, the tumor is classified as a mixed mesenchymal sarcoma.

Before entering upon an exposition of the individual members of this family of sarcomas, I propose to discuss two benign tumors that bring the basis of the classification into sharper focus. The first case* is that of a spherical, sharply demarcated tumor of the uterine wall, grossly distinguishable from he usual leiomyoma by its softer consistency and yellowish color. Microscopic examination revealed it to be composed of approximately equal parts of mature smooth muscle and mature fat. It is, if you like, a lipomyoma or a ipoleiomyoma. However, it also can be considered a benign mesenchymoma; he term mesenchymoma was devised by Stout[12] to include neoplasms composed of a mixture of more than one connective tissue element. Both benign and malignant varieties have been described. It is evident that in this case he fat is heterologous to the uterus. Recent reports of uterine lipomas by Decker *et al.*[13], Bickel and Bennett,[14] and Chachutow and Brill[15] indicate that uch tumors may be "pure," composed entirely of adult fat, or "mixed," composed of both fat and smooth muscle. Historical opinion is divided into two chools concerning the problem of the origin of the heterologous fat: displaced mbryonic rests versus metaplasia from smooth muscle.

The second case is a benign papillary adenofibroma of the endometrium that occurred in an elderly woman, giving rise to bleeding and necessitating hysterectomy. It was composed of a central fibrous stalk covered by layers of proliferated but innocuous-appearing endometrial stroma that were in turn nvested by a single layer of glandular and surface epithelial cells which dipped own into the clefts formed by the ramifying architecture of the fibrovascular troma. There was no cellular pleomorphism and no heterologous tissue. This was a mixed tumor composed of homologous tissues, the benign counterpart of a homologous carcinosarcoma.

The pure homologous tumors recently have been reviewed by Ober and

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* Courtesy of Peter Gruenwald, Margaret Hague Maternity Center, Jersey City, N. J.

Jason[16] and by Symmonds and Dockerty.[9] While some growths are exophyti with limited invasive activity, others may be extremely invasive and give ris to both pelvic and distant metastases. In general, the degree of malignanc of endometrial stromal sarcoma is less accurately predicted from close examina tion of its cellular features than from careful gross and microscopic evidenc of invasiveness. An unusual member of this series is the so-called stromatou endometriosis (or endolymphatic stromal myosis), a proliferative lesion o endometrial stroma which has a limited capacity for autonomous growth. O the 43 cases summarized by Park,[17] only 7 were considered to have metastasized and of these, only 2 exemples were histologically proved. Many cases surviv for long intervals; for example, the case described by Hunter *et al.*[18] survived for 26 years with recurrent and metastatic tumor. The one case in the Sloane-Delafield series died of unrelated disease 16 years after removal of the primary tumor and 13 years after excision of a vaginal metastasis. The cases in th files of the AFIP are for the most part still *sub judice*. Occasionally, on encounters a case that appears to lie midway between diffuse sarcoma of the endometrial stroma and stromatous endometriosis. For example, the bul of the tumor in one such case appeared as a pure stromal sarcoma. However at its invading edge there was evidence of the distinctive pattern of endolymphatic invasion. This patient is alive and well four years after surgical extirpation. Whether or not the endolymphatic pattern should be construed as evidence of reduced malignancy is an important question that remains to be solved. Dockerty and his colleagues classify stromatous endometriosis as a sarcomalike lesion and, until more cases are followed to a determinate end point, one cannot say more. In some cases of stromatous endometriosis, one can see an epitheliumlike pattern differentiating from the matrix of "juvescent" stromal cells, and on occasion peculiar vascular patterns may be observed. This coincides with Gruenwald's observations of the lability of the stroma-epithelium relationship.

The pure heterologous tumors, as mentioned above, differ chiefly in a quantitative way from mixed mesenchymal sarcomas. Rhabdomyosarcoma is the most frequent member of this group. Kulka and Douglas[19] recently reviewed this lesion and accepted 19 prior reports in addition to their own. Most of the cases proved to be fatal within a year; a minority of cases were not reported with follow-up data. The one case we accept as a "pure" rhabdomyosarcoma is still *sub judice*, but shows clinical evidence of metastasis less than three months after operation. Kulka and Douglas point out a relatively high coincident occurrence of an independent adenocarcinoma elsewhere in the female genital tract in these patients. One of the cases on file at the AFIP* (not included in the tabulation) is a collision tumor in which a pure rhabdomyosarcoma and a pure adenocarcinoma, both of endometrial origin, collide at one point, a single low-power field in diameter. It is possible that this observation is of significance in the pathogenesis of such lesions.

Chondrosarcoma is even less common than rhabdomyosarcoma. Less than 10 acceptable cases have been reported. The most recent case is that of

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* Courtesy of Gretchen V. Squires, Baptist Hospital, Pensacola, Fla.

Pena,[20] in which large spherical nests of neoplastic cartilage were separated by extremely narrow bands of nonspecialized sarcoma. The patient died eight months after hysterectomy, with mediastinal and pulmonary metastases composed entirely of stellate mesenchymal sarcoma without any cartilage in the metastases. The solitary uterine chondrosarcoma in the AFIP files exhibited a similar phenomenon—almost no "stromal sarcoma" in the primary growth, but no cartilage in the metastases. The extent to which the heterologous elements found in such tumors reflect local environmental factors that promote cell specialization along a given pathway remains debatable.

Osteosarcoma is likewise rare. I have been able to find only two cases so labeled in which I was satisfied that osseous elements were predominant and in which no other heterologous element was present; these are the cases reported by Scheffey *et al.*[21] and by Stier and Lyman.[22] Both of these were thought to have arisen in pre-existing leiomyomas, Scheffey's case following irradiation therapy. The AFIP files contain a case in which osteoid tissue is very prominent, but anamnestic data are not yet complete.

Liposarcoma is so rare that no unequivocal case has yet been reported as being primary in the uterus. Fat occurs rarely as a component in mixed tumors, but in general it is difficult to be certain whether fat in a rapidly growing neoplasm is due to cellular degeneration or whether the cells are truly lipoblastic.

The mixed sarcomas comprise about 60 per cent of the group of mesenchymal sarcomas. Mixed tumors include carcinosarcoma, mixed mesenchymal sarcoma, and sarcoma botryoides. The first two types occur chiefly in the uterine corpus in postmenopausal women; sarcoma botryoides occurs chiefly in infants and children, usually in the vulvovaginal region, less often in the cervix of younger women, and rarely with its distinctive gross appearance in the corpus uteri. Most commonly, sarcoma botryoides contains some heterologous element and is best considered the juvenile version of a mixed mesenchymal sarcoma; however, in a few cases heterologous elements are not demonstrable and, in a very few cases, chiefly in the older age groups, neoplastic glands have been reported. Precisely where one draws the line between a carcinosarcoma with a complex polypoid pattern and a botryoid sarcoma is a matter of individual taste; I am inclined to classify all tumors with both neoplastic glands and neoplastic stroma as carcinosarcomas.

In carcinosarcoma the neoplastic epithelium may be glandular, squamous, or a mixture of the two. In any case, it is homologous to the uterus. One of the cases in the AFIP series arose in the cervix and was composed of an intimate admixture of squamous carcinoma and stromal sarcoma consisting of elongated bipolar cells arranged in interlacing bundles. In the other cases, almost all arising in the corpus, the epithelial component was glandular; in a few instances adenoacanthomatous foci could be found and, in a rare instance, discrete nests of keratinizing squamous carcinoma could be seen without direct contact with areas of glandular carcinoma. It is only in the neoplastic stroma that heterologous elements occur. This is most frequently seen in the presence of rhabdomyoblasts. Areas of cartilage are next most frequent;

osteoid and well-formed bone are uncommon, and fatty elements are not onl infrequent but difficult to evaluate. I doubt that the exact details of cellula composition bear any recognizable relationship to the clinical course.

Study of the metastases of carcinosarcomas shows that the predominan element to metastasize is the epithelial component, that is, most of them wil metastasize much like an adenocarcinoma of the endometrium. However, i is not uncommon to find that the metastases may resemble the original tumo and contain an admixture of carcinoma and sarcoma. Less frequently one can find isolated metastases of sarcoma without carcinoma.

Meyer[23] classified mixed tumors into three groups: (1) collision tumors which originate from accidental collision of two originally discrete or separate tumors; (2) combination tumors, in which the single essential parts have a common histogenesis, that is, a common ancestor, the combination meaning a union with various possibilities; and (3) composition tumors, which are mixed tumors in which both the stroma and glands are blastomatous without being related histogenetically. Clearly, our concept of carcinosarcomas places them in the group of combination tumors. The occasional occurrence of collision tumors emphasizes the need for carefully selected, well-oriented blocks for study.

Mixed mesenchymal sarcoma is morphologically similar to carcinosarcoma except for the lack of neoplastic epithelium. As previously stated, one or another heterologous element may be dominant in this group. For example, I should reclassify Hartfall's[24] cases of chondrosarcoma as mixed mesenchymal sarcomas, but the value of such distinctions remains to be proved. In general, a mixed mesenchymal sarcoma contains a relatively prominent proportion of stromal sarcoma, usually showing both cellular and myxomatous areas, plus an admixture of varying amounts of neoplastic cartilage, bone, and osteoid neoplastic striated muscle. In one case in the Sloane-Delafield series the mixture was of leiomyosarcoma and rhabdomyosarcoma. Alzanauer[10] has summarized the reported autopsied cases; in general, the predominant component was "undifferentiated cellular sarcoma"; pelvic recurrence and metastasis were most frequent, while peritoneal implants and metastases to the lung and pleura and to lymph nodes were only slightly less frequent. None of the cases survived longer than 24 months.

One of the best documented cases in this group is reported by Lebowich and Ehrlich.[25] In their discussion they state that the diagnosis cannot be made in the absence of embryonal myoblasts. I should not go quite so far. When present, they are extremely useful because their presence disposes effectively of the argument that such a tumor is merely a carcinoma that is poorly differentiated and growing in a "spindle-cell" pattern with no attempt at gland formation. However, the basic element in all mesenchymal sarcomas is the presence of neoplastic mesenchyme, not the heterologous tissues that may differentiate from it.

With respect to sarcoma botryoides, I have little to add to statements intercalated elsewhere in the text. Simpson[26] has reviewed thoroughly the cases arising in the cervices of women in the stage of active reproductive life. Recent reports by Duckett *et al.*[27] and Cavins *et al.*[28] add a few more well-documented cases occurring in infants. The rarity with which this tumor is encountered in practice is reflected by the fact that the one case in the Sloane-Delafield series is the first case to be seen primarily at Babies' Hospital, New York, N. Y., and to be so diagnosed. The fact that, in infants and children, this tumor is likely to present itself externally rather early in its history has prompted surgeons to treat a number of these patients by extensive extirpative procedures; in some instances their efforts have been apparently successful. Two cases reported by Ober, *et al.*[29] were present at birth and were treated by abdominoperineal hysterocolpectomy; both infants have survived for more than 2½ years without recurrence.

Related histogenetically and histologically to mixed uterine sarcomas are the neoplasms of the subcoelomic mesenchyme, a tissue derived from the mesenchyme of the urogenital ridge and distributed in the adult female pelvis as an inconspicuous layer of cells beneath the peritoneal mesothelium covering the uterus and its ligaments, the fallopian tubes, ovaries, dome of the bladder, and pelvic parieties. It is this tissue which forms pelvic adhesions, serves as the site for ectopic decidual reactions, and bears a distributional relationship to endometriosis. The occurrence of an endometrial stromal sarcoma[16] in a case of pelvic endometriosis of long duration was followed by the development of multiple peritoneal "implants," and the question of multicentric origin of neoplasia in extrauterine as well as intrauterine endometrium was raised. Subsequently, MacFarlane and Pritchard[30] reported a case of carcinosarcoma arising in an endometrial cyst of the ovary; Ober and Black[31] reported two more cases of periuterine sarcoma associated with endometriosis. More recently, a well-documented case of sarcoma arising in the stroma of an endometrial cyst of the omentum has been reported by Ginzler and Herrera.[32]

Vascular tumors of the female genital tract have been the subject of a recent review by Gerbie *et al.*,[33] who were able to find reports of 12 benign hemangiomas of the cervix and an indeterminate number, probably less than 20, of the corpus. I have seen only one example of a hemangioma of the cervix, an incidental and asymptomatic finding. Only 1 lymphangioma of the cervix has been reported, and possibly 3 of the corpus.* Loeffler's[34] account of a lymphangioma of the corpus is well documented.

The malignant counterparts of these tumors, the hemangiosarcoma (hemangioendothelioma) and lymphangiosarcoma (lymphangioendothelioma) are extremely rare. No more than a half dozen hemangiosarcomas of the uterus have been reported. The only unequivocal case I have seen is in the Sloane Hospital series and was reported previously by Stout.[35] It arose in the endometrium of a 28-year-old woman whose menorrhagia was not controlled by two curettages. At hysterectomy the true nature of the tumor was determined; it had invaded the myometrium. The patient died of generalized metastases 14 months later. Only two cases of lymphangiosarcoma of the uterus are reported; I have never seen one.

Recently, some attention has been focused on hemangiopericytoma of the uterus. The first report of this tumor in the uterus was by Greene and Gerbie,[3] who presented five well-illustrated and well-documented cases. In general these seem to be discrete, well-demarcated, spherical tumors, grossly similar to leiomyomas, although possibly with a less whorled texture. They are more cellular than the average myoma. Microscopically they exhibit a proliferation of medium-sized ovoid perivascular cells, presumably derived from the pericytes of Zimmerman. Reticulum stains to demonstrate the single layer of endothelial cells on an argyrophilic membrane are useful in distinguishing this type of tumor from proliferations of endothelial origin. The perivascular pattern is quite distinctive.

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* Following the completion of this paper, a well-documented account of the first glomus tumor to be found in the uterus was published by Borghard-Erdle and Hirsch.[43] This represents an additional variety of benign tumor of vascular origin.

In general, hemangiopericytomas are unpredictable in terms of local recurrence and metastasis. None of the cases described so far in the uterus has displayed invasive or recurrent potentialities. I suppose that a circumscribed tumor within the myometrium is more likely to be benign than, say, a tumor of similar histological appearance that is locally infiltrative in soft tissue.

Pedowitz *et al.*[37] have reported four possible examples of hemangiopericytoma of the uterus, emphasizing the possible relationship of this tumor to stromatous endometriosis. The evidence is far from conclusive and seems to revolve around the degree of relationship between the pericyte of Zimmerman and the endometrial stromal cell. I doubt that anyone would care to commit himself to a definitive statement in this connection at this time.

Malignant lymphoma in the female genital tract poses the unanswerable question of primary site versus multicentric origin. In cases of leukemia, the female genital organs are found post-mortem to be involved by leukemic infiltrates in proportion to the assiduity with which search is made for such manifestations. Occasionally vaginal bleeding may be a presenting symptom of an underlying leukemia of lymphomatosis; the diagnosis may even be established by cervical biopsy[38] or by curettage.[39] Very rarely a lymphoma may appear to be primary in the uterus, as in one of the uterine lymphosarcomas reported by Walton,[40] or in the reticulum cell sarcoma currently being reported by Weiss and Matthews.[41] The clinical and pathological aspects of this problem recently have been reviewed by Johnson and Soule,[39] who survey the literature and add five cases of their own. There are less than twenty cases in all; in most of them the uterine lymphoma was part of generalized disease, which was present concurrently or manifested itself soon thereafter.

A somewhat different problem is presented by the very infrequent example of reticulum cell sarcoma of the vulva,[14, 40, 41] which is a true example of reticulum cell sarcoma primary in the soft tissue. I have been able to find no satisfactory example of Hodgkin's disease occurring in the uterus either primarily or secondarily.

Finally, we come to the two groups listed as metastatic sarcoma and as "unclassified" sarcoma. I have never seen or heard of sarcoma metastatic to the uterus, unless one includes leukemic infiltrates or multicentric lymphoma. The group of unclassified sarcomas is placed in the table both as a warning and as a safety valve. No sooner does the unwary taxonomist commit himself to print and present a supposedly complete classification of a given entity than he receives a case that beggars description and resembles nothing he has ever seen before. I have seen such cases. For example, Case 1 in the communication by Bickel and Bennett[14] looks very much like a giant cell tumor of tendon sheath of fascial origin; alternatively, it might be interpreted as a leiomyoma showing osteoid metaplasia and osteoclastic activity, or even as a uterine metastasis from an occult primary tumor in bone. I am frank to confess I have no idea what it is. Likewise, a recent case reported by Schulz[42] deals with a pigmented uterine neoplasm similar to the "neural crest" tumor or melanotic progonoma, not a sarcoma but derived from neuroepithelium. I concur in Schulz's diagnosis, but I should have been hard put to make it myself. I still cannot imagine how the neural crest derivatives entered the uterus. There are several cases in the files of the Armed Forces Institute of Pathology, of course, which neither Hugh Grady, nor I myself, cares to classify definitively.

Let us take comfort in a story Alfred Plaut tells about Aschheim. Apparently, a young pathologist brought a set of slides to Aschheim; let us pretend they were from a most unusual uterine tumor. Aschheim looked at them long and hard. Finally he turned to the young man with a smile of apparent insight and illumination, and said, "A splendid case, indeed! Shall I put it in the Lorelei file or in the Lohengrin file?" The young pathologist was perplexed and accepted the gambit. "Why, what do you mean?" he asked. Aschheim replied, "The Lorelei file is: '*Ich weiss nicht was soll es bedeuten* (I know not what it means).' The Lohengrin file is: '*Nie sollst du mich befragen!* (Don't ever ask me).' " I think we had better let it go at that.

Acknowledgments

Most of the material that serves as the basis for this paper was collected in two large series, one at the Armed Forces Institute of Pathology, Washington, D. C., with Hugh G. Grady, and the other at the Sloane Hospital for Women and Francis Delafield Hospital, New York, N. Y., with Harold M. M. Tovell. In addition, numerous pathologists who have learned of my special interest in this family of tumors have been generous enough to place their files at my disposal, and I am grateful to them for the opportunity to fill in lacunae in the two larger series, as well as for enriching my own experience.

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ESTROGENS, ENDOMETRIAL HYPERPLASIA, AND ENDOMETRIAL CARCINOMA

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The subject of this paper can be stated very simply: are estrogens an important and common etiological factor in the development of endometrial carcinoma in the human? Such a relationship is certainly inferred or suggested in numerous publications.

The following is a quotation from Willis' book *The Pathology of Tumors* (1948): "The obvious relationship of hyperplasia and neoplasia in certain cases, however, must not be allowed to mislead us. There are legion (sic) hyperplasias which never end in tumor formation and there are many tumors which develop without any recognizable previous hyperplasia. Clearly, then, the causation of a tumor involves more than the mere persistence of hyperplasia evoking stimuli. The specific nature of the stimulus is all-important; for example, on the experimental side both carbon tetrachloride and aminoazotoluene cause liver damage followed by regenerative hyperplasia but tumor formation is rarely seen following the former and very frequently following the latter."

Prior to pursuing this subject further it seems prudent to define our own present position on this subject. We believe that some (and probably the minority) of endometrial carcinomas are preceded by and possibly induced in or developed from areas of endometrial hyperplasia. The exact initiating factor in such instances is unknown to us, but certainly there is evidence that endometrial carcinoma has developed in patients with past evidences of hyperplasia (Hertig and Sommers, 1949; Speert, 1952).

Next, it is essential to define what is meant by hyperplasia. Pathologically, the definition is self-explanatory: an increase in size of an organ due to reduplication of cellular elements. To our way of thinking this presupposes or necessitates the presence of at least a few mitotic figures. In addition, in the endometrium there is usually variation in size and shape of the glands and pseudostratification of the nuclei. This latter phenomenon should not be confused with early atrophic changes in which (as in the myometrium) there is a loss of cytoplasm and apparent crowding together of nuclei which, in endometrial glands, can produce an appearance of pseudostratification. Actually, this marked diminution in cytoplasm and lack of mitotic figures clearly distinguish this atrophic condition from hyperplasia.

It is unfortunate that the terms "Swiss cheese" and "cystic" hyperplasia of the endometrium were ever suggested. Hyperplasia may or may not include an appreciable distention of the glands, but cystic distention of glands is not of itself evidence that hyperplasia is present. The increasing frequency of cystically distended glands with advancing postmenopausal years will be discussed later.

We are more than a little unclear as to the exact status of the stroma with hyperplasia. Certainly with active hyperplasia (estrogen induced) stromal mitoses are found. We suspect that in early hyperplasia the stroma is moderately edematous, but that soon the stroma becomes compact.

We are uncertain if (and at this time unable to understand how) estrogen-induced hyperplasia could act selectively either on glands alone or on stroma alone. We are therefore unwilling to grant that there are such entities as estrogen-induced "stromal hyperplasia" or "pure glandular hyperplasia." True, we have seen focal areas of so-called glandular hyperplasias (with some amount of intervening stroma between glands and normal glandular cytology), but we believe that these are actually benign adenomas. It has always seemed a paradox that benign adenomas are described in all other glandular organs, but are usually considered not to exist in the endometrium.

Finally, we wish to emphasize (as have others) the difficulty, on rare occasions, in distinguishing marked forms of hyperplasia from well-differentiated carcinoma. As a relevant aside, we have been confused on occasion by photographs appearing in various publications attempting to relate endometrial hyperplasia to carcinoma. The confusion has arisen because of our difficulty in distinguishing, in some instances, where the hyperplasia ended and the carcinoma began. In other instances we were uncertain as to whether carcinoma was present and, in still others, whether hyperplasia was present.

Have Administered Estrogens Produced Endometrial Carcinoma?

Experimental animal. Data on this subject are given in detail in preceding articles in this monograph. We propose to emphasize a few points. To the best of our knowledge, carcinoma of the endometrium has been produced by administered estrogen (alone, and not with a true carcinogen) in one species, the rabbit, and by one group of workers (Meissner *et al.*, 1957). In a few reviews that we have read there have been inferences that endometrial carcinoma has been produced by prolonged estrogen stimulation in the mouse and in the rat. In the mouse, Hall *et al.* (1953) inserted 20-methyl cholanthrene in the uterus, and the animals were subsequently given stilbesterol. These investigators conclude that the estrogens "acted as a co-carcinogen, accelerating induction and perhaps stimulating growth." No data are given as to what happened with the 20-methyl cholanthrene alone. Other tumors in the mouse uterus have been produced by the prolonged action of estrogens (Gardner and Ferrigno, 1956). These tumors, however, are of the serosa—at least, they do not involve the endometrium. They bear a certain degree of resemblance to the human mesothelioma occasionally found in the uterus. The data are concerned with the rat report on endometrial carcinoma arising in one animal whose hypophysis was "masculinized" by implanting a testis subcutaneously shortly after birth (Pfeiffer, 1949). After maturity such animals are in practically constant estrus, with continual estrogen production. This case seems authentic, but one wonders how many animals (perhaps hundreds) similarly treated did not develop adenocarcinoma of the endometrium. The data in primates treated with estrogens for several years with production of no such lesion should be mentioned (Engle *et al.*, 1943; Pfeiffer and Allen, 1948).

Human. Sporadic reports have appeared concerning endometrial carcinoma appearing during the course of estrogen therapy (Novak, 1951; Gemmel and Jeffcoate, 1939; Freemont-Smith, 1946; and others). However, such coincidental occurrences must be expected. The number of women given estrogens in this country is not known to us, but we strongly suspect that the number of estrogen-treated women developing cancer of the endometrium is no greater than the expected incidence for this particular group. Certainly, evidence to the contrary has not been adduced.

Feminizing Tumors and Endometrial Carcinoma: Cause and Effect or Coexistence?

Personal experience. In the files of the departmental laboratory at Northwestern University, 55 feminizing tumors are listed. Two of these tumors were associated with adenocarcinoma of the endometrium. The first had received intrauterine radium 4 years prior to the definitive diagnosis and surgery (Gardner *et al.*, 1957). There was no uninvolved endometrium in the blocks, but the tubal epithelium and the stratified squamous epithelium of the cervix both showed evidence of estrogen stimulation. Incidentally, there was much intracellular fat in the granulosa-theca cell tumor. The second was also an undoubted adenocarcinoma. The ovarian tumor was diagnosed by the authors and by others as a granulosa cell tumor of the ovary. Although the fat stain was completely negative, the uninvolved endometrium showed hyperplasia.

These 2 of 55 constitute a 3.6 per cent association between feminizing tumors of the ovary and adenocarcinoma of the endometrium. For reasons to be discussed later, we do not believe that these data can be used as valid evidence that feminizing tumors produce adenocarcinoma of the endometrium.

The "pro" data. Many articles in the literature infer or "indicate" that feminizing tumors produce prolonged and excessive amounts of estrogen. This in turn produces hyperplasia, which is followed by carcinoma of the endometrium. These articles have been reviewed in detail by Larson (1954). Dockerty (1940) and Dockerty and MacCarty (1939) reported the coexistence of 3 endometrial carcinomas with 40 feminizing tumors of the ovary (7.5 per cent) and suggested that the estrogen from the ovarian tumor might have caused the uterine carcinoma. By 1951 (Dockerty and Mussey) the Mayo Clinic group had enlarged their series to 87 feminizing tumors. Sixteen of these (18.4 per cent) were associated with endometrial carcinoma. The significance of this marked increase in association will be commented on later.

Other workers have reported a similar high association. For example, Kottmeier (1947) found carcinoma of the endometrium in 16 per cent of patients with granulosa-theca cell tumors of the ovary, and Ingram and Novak (1951) in 12 per cent of postmenopausal women with feminizing tumors of the ovary. Mansell and Hertig (1955) reported 80 instances of feminizing tumors of the ovary, in 75 of which the endometrium was available, with 15 per cent showing endometrial carcinoma. In patients over 50 the incidence was 24 per cent. More recently, Greene (1957) has reviewed the literature and the material of the Ovarian Tumor Registry of the American Gynecological Society, St. Louis, Mo. In this material he found records of 289 feminizing tumors. Fifty-six of these had accompanying endometrium. Thirteen of the latter (23 per cent) showed endometrial carcinoma. The validity of these particular data will be discussed later. Greene, incidentally, found in the literature 132 instances in which feminizing tumors of the ovary and adenocarcinoma of the endometrium were coexistent (one wonders how many thousands of feminizing tumors have not been reported because they were not associated with adenocarcinoma of the endometrium and, hence, were not considered "interesting").

The "anti" data. The material accumulated in our laboratory has already been mentioned. Emge (1953), wishing to avoid the matter of interest bias and to obtain a statistically valid sample, wrote to the heads of the departments of obstetrics and gynecology of various medical schools and medical centers. From the data thus obtained he was able to report on 753 feminizing tumors of the ovary, of which 25, or only 3.3 per cent, had adenocarcinoma of the endometrium. In addition, he was personally able to report 43 feminizing tumors, none of which were associated with endometrial carcinoma.

In a review of the literature on this subject, Larson (1954) was able to find reports on 919 feminizing tumors, of which 4.8 per cent were associated with endometrial carcinoma. Broken down as to "genital age," this included an incidence of 1.3 per cent in premenopausal patients, 10.3 per cent in postmenopausal, and 3.9 per cent in women of unknown or, rather, unreported age.

Evaluation of data. The evaluation of data is extremely difficult. Women in Boston, Baltimore, or Rochester should not vary significantly in this regard from women in San Francisco or in Chicago. Necessarily, then, the source of material and/or interpretations in these various centers must vary. Undoubtedly this last factor is important. For example, in our own series of 55 feminizing tumors we recognize 2 adenocarcinomas. According to our cross file, a third tumor is listed as being associated with "possible hyperplasia of the endometrium, possible well differentiated adenocarcinoma of the endometrium." The original diagnosis was not made by any of us and, in reviewing the slides, we do not believe adenocarcinoma to be present --only hyperplasia. It is possible that other workers would consider carcinoma to be present. This would then increase our association figure to 5.5 per cent.

An example of what must be categorized as changing criteria for the diagnosis of adenocarcinoma can be seen in the various Mayo series. Originally they reported a 7.5 per cent association (3 of 40 patients). Eventually they reported adenocarcinoma in 16 of 87 patients. This means that in their last 47 patients their incidence increased to 27.7 per cent almost a 400 per cent increment. The odds against this change being due to chance by the chi square method are nearly 100 to 1.

Another cause for such a variation in the reported association between these 2 tumors could be labeled as "institutional bias." In at least 1 series (Ingram and Novak, 1951) from one of the more prominent centers, 2 of the 4 cases included were received from outside sources. Certainly one would be more inclined to send in the interesting combination of feminizing tumor of the ovary and adenocarcinoma of the uterus rather than a run-of-the-mill feminizing tumor of the ovary alone.

This leads to a similar interest bias in the general literature. Obviously there would be a greater tendency to report those cases with such an interesting tumor combination in preference to the uncomplicated type, which leads to the astonishing fact that, in 289 feminizing tumors sent to the American Gynecological Ovarian Tumor Registry, associated endometrial carcinoma was found in only 13, or 4.5 per cent. It is granted that endometrium was available for study in only 56 cases, but one must remember that the Registry does have a follow-up and probably would later pick up many if not most of the adenocarcinomas present but not diagnosed because no endometria were available for study with the first set of sections.

Finally, we should give consideration to Larson's suggestion that the expected incidence of multiple tumors in any one individual is so high that we should expect an equally high association rate with these particular tumors. Larson reviewed the literature on this subject thoroughly and pointed out that the incidence of multiple tumors in general is approximately 5 to 7 per cent. With 1696 cases of endometrial carcinoma it was 6.3 per cent. More recently, Liu (1956) has shown (in her incidental data) that in 460 patients with breast carcinoma there was carcinoma in another site in 7 per cent. Even more recently, Sommers and Meissner (1957) reported that, in 38 autopsied patients with endometrial carcinoma, 14 (37 per cent) had cancers in other organs, namely, breast, cervix, colon, ovary, lung, bile duct, and apparently no feminizing tumors of the ovary.

Summary. We believe that the data on the co-existence of feminizing tumors of the ovary and endometrial carcinoma are regional, contradictory, and confusing. Our evidence shows that feminizing tumors only rarely produce prolonged excessive estrogens that may induce hyperplasia of the endometrium and may eventuate in carcinoma. However, when these two tumors do co-exist, it would be interesting to know the answers to the following two questions:

(1) Was the feminizing tumor present and producing excessive estrogens 8 to 10 years prior to the diagnosis of the adenocarcinoma of the endometrium? Obviously, except by clinical inference it would be impossible to answer this question, but certainly the protagonists should at least attempt to show a prolonged antecedent history of abnormal premenopausal bleeding or of postmenopausal bleeding.

(2) Of less, but at least of suggestive importance: at the time of operation was the "feminizing" tumor producing any estrogens or sufficient estrogens to produce stimulatory effects? In probably one half of the instances (as blocks are usually cut) noncancerous endometrium is not present. Even if this index of measuring estrogenic effect is not included, the status of the endosalpingeal epithelium and the stratified squamous epithelium of the cervix can and does indicate whether enough estrogen is or is not being produced to have a biological effect.

These two not necessarily impertinent questions are asked because we are not happy with the commonly accepted concept that yellow color and intracellular fat necessarily mean hormonally active tumors. These criteria certainly have proved to be less than universally valid in our own experience.

Hyperplasia and Endometrial Carcinoma

Our definition of hyperplasia has been given in the introduction. Reference has been made to the fact that cystic distention of glands does not, of itself, mean hyperplasia. In fact, a recent study by Parks *et al.* (1958) confirms Speert's (1949) finding that cystic dilatation of glands in postmenopausal women is a retrogressive phenomenon caused by blockage of gland orifices, with subsequent distention due to the slow accumulation of fluid.

It is commonly accepted that hyperplasia is a fairly frequent phenomenon in the postmenopausal female. Actually this is not true. The belief is probably based on the following factors:

(1) Specimens are examined from women subjected to dilatation and curettage because of postmenopausal bleeding. Naturally one would expect a high incidence of hyperplasia and/or other abnormalities in such a group of patients.

(2) Specimens are examined from women subjected to a dilatation and curettage prior to a vaginal procedure, not including a hysterectomy, and done for the correction of prolapse and/or symptomatic cystocele. In such instances, with truly atrophic endometrium, frequently no tissue is obtained. Such cases are omitted from the calculated results. An example of the latter is a study by McBride (1955) in which 1524 postmenopausal women were subjected to curettage. In 1315 only mucus was obtained. Actually, McBride's data were based on the tissue obtained in the remaining 206 patients. Such a study naturally gives an artificially high incidence of active or hyperplastic endometria.

(3) The habit of equating cystic dilatation with hyperplasia leads to misinterpretation. An example of this is the report by Novak and Yui (1936) pertaining to the high incidence of hyperplasia associated with endometrial carcinoma (practically all patients being postmenopausal). TeLinde (Novak and Yui, 1936), from the same institution, discussed this paper and believed that the changes reported as hyperplasia merely represented retention cysts, which are very common in postmenopausal endometria. Another example is the publication of Fahlund and Broders (1946), in which they compared the findings in the uninvolved endometria of patients suffering from carcinoma of the endometrium with those of patients of similar age but free of carcinoma of the endometrium. They did not use the word "hyperplasia," but they found an inordinately high incidence of active endometria, early and late proliferative. Apparently endometria with a moderate number of cystic glands were classified as early proliferative, and those with many cystic glands as late proliferative.

The whole subject of the endometria of normal postmenopausal women has recently been reviewed and studied by Parks *et al.* (1958). That study was limited to uteri from 335 women who were at least 2 years postmenopausal, had no history of bleeding, had not received estrogens, and most of whom had been subjected to a vaginal hysterectomy as part of a procedure for repair of a prolapse and/or cystocele. The endometrial findings are summarized in TABLE 1. It seems evident that, in normal postmenopausal women who have not received estrogens and who are not having postmenopausal bleeding, evi-

TABLE 1

INCIDENCE OF ENDOMETRIAL FINDINGS IN NORMAL POSTMENOPAUSAL WOMEN

Years post-menopausal	No. of specimens	Simple atrophic (%)	Cystic atrophic (%)	Hyperplastic (%)	Proliferative (%)
2–10	170	62.5	25.7	1.2	0.6
11–20	123	38.0	61.2	0.8	0.0
21+	42	32.5	67.5	0.0	0.0

dences of estrogen stimulation of the endometrium are practically nonexistent. It might be justifiable to note here that women of this age group are those in whom carcinoma of the endometrium develops most frequently. It should also be added that such lack of evidence of endometrial stimulation does not coincide with data obtained from studies of the cytology smears from normal postmenopausal women as presented by Randall *et al.* (1957). The explanation for this difference is unclear. Possibly it can be explained by the fact that the vagina is a more sensitive index of estrogenic stimulation than the endometrium, and (2) the vagina's cytology may be influenced by various traumatic influences (for example, coitus, douches, prolapse) that per se cause cornification. Therefore, vaginal smears may not be too accurate as an indicator of endogenous estrogen production. We do not know whether either of these suggestions is correct, but we are definitely certain that the endometria in the vast majority of women in whom endometrial carcinomas may later develop do not show evidences of estrogenic stimulation. We wish to reaffirm the fact the endometria studied by Parks *et al.* (1958) came from *normal* (nonbleeding), nonestrogenically treated women, because in another series reported by Roddick and Greene (1958a) the incidence of postmenopausal bleeders and estrogenically treated women was relatively high, and the endometria showed a much higher incidence of stimulation.

Coexisting hyperplasia. There is, of course, an error in logic in articles having to do with this subject. Data showing a coexistence of carcinoma with hyperplasia tell us nothing as to the status of the endometrium at the time of the inception of the carcinoma. Nevertheless, such data are frequently quoted and were examined in some detail in Larson's review. Only the highlights of this data will be mentioned here.

Probably the first publication that claimed a high incidence of hyperplasia and endometrial carcinoma was that of Novak and Yui (1936). These investigators reported 39 per cent incidence of hyperplasia in the uninvolved endometrium of patients with endometrial carcinoma (TeLinde's comments in his discussion of this paper have already been quoted). In 1948 Speert reviewed the data on patients with endometrial carcinoma at Roosevelt Hospital, New York, N. Y. He stated that unequivocal hyperplasia was fairly common, but gave no data, since he had "no adequate controls." He did, however, publish a photograph (Speert, 1948, figure 1, page 589) labeled as adenocarcinoma with adjacent hyperplasia. In our opinion the latter is obviously an area of cystic atrophy and not hyperplasia. Those who are incredulous should compare this photograph with those in Speert's excellent article having to do with the "Endometrium in Old Age" (1949).

Other authors have claimed a high association between endometrial carcinoma and hyperplasia. For example, Bamforth (1956) found "cystic hyperplasia" in 17 of 22 cases with uninvolved endometrium. The obverse is also reported. Waschki (1955) found glandular cystic hyperplasia in only 8 of 138 (5.8 per cent) specimens and stated that there was no direct connection between the 2 lesions. Jones and Brewer (1941) found hyperplasia in only 2 of 68 patients. McBride (1956) had 56 specimens with uninvolved endometrium; in only 8.8 per cent was there hyperplasia.

Data on this subject were available from 40 patients in the series reported by Roddick and Greene (1957). In addition, slides from 80 others of the more recent carcinomas of the endometrium processed in our departmental laboratory were pulled from the files and examined hurriedly by one of us (RRG). None had had radium treatment. Sixty of the total had uninvolved endometrium. The findings are shown in TABLE 2. Only 10 (16.6 per cent) showed hyperplasia, and one of these was in the inactive or regressive phase.

Another technique for attacking this subject is to study all the hyperplasias and note how many are associated with endometrial carcinoma. Payne (1937) studied 534 specimens of hyperplasia, 496 from premenopausal and 38 from postmenopausal women. Associated endometrial carcinoma was found in 1.6 per cent in the premenopausal group and in 10.5 per cent in the postmenopausal group. Payne was not impressed with the possibility of such a relationship between hyperplasia and carcinoma. He noted also that one would expect any such association to be more common in the postmenopausal women than in premenopausal, since the former were in the age group in which endometrial carcinoma commonly occurs.

Pre-existing hyperplasia. Two types of studies have been made in pursuing this subject—prospective and retrospective studies. In the former, patients in whom a diagnosis of hyperplasia had been made are followed to see if endometrial carcinoma subsequently develops. In the latter, earlier dilatation and curettage or endometrial biopsy specimens from patients who now have endometrial carcinoma are sought and studied.

Prospective studies. This subject was reviewed in some detail by Greene and Brewer (1941), and the old literature will not be repeated here except to note

TABLE 2

STATUS OF UNINVOLVED ENDOMETRIUM WITH CARCINOMA OF THE ENDOMETRIUM

Number of patients with carcinoma		120
Number of specimens with uninvolved endometrium		60
Number of specimens with normal endometrium		50
Secretory	5	
Proliferative	6	
Atrophic	39	
Number of specimens with abnormal endometrium		10
Hyperplasia	9	
Regressing hyperplasia	1	

that there was a surprising lack of carcinoma subsequently developing in patients who had been followed because of an earlier diagnosis of hyperplasia.

More recently, Emge (1950) found that 4 of 374 patients with endometrial hyperplasia subsequently developed endometrial carcinoma. Waschki (1955) followed 1262 patients with hyperplasia (16 with atypical, adenomatous changes) for variable periods of time. None of these developed carcinoma. Hall (1957) was able to follow 151 patients for a minimum of 10 years (except those that developed carcinoma prior to this time). His total group, however, included 58 whom most pathologists would consider as having at least "carcinoma *in situ*." His subgroups Ia and Ib correspond to the usual definition of hyperplasia and contain 93 patients. Three subsequently developed adenocarcinoma after 9, 16, and 16 years.

Retrospective studies. These include the classic studies by Hertig and Sommers (1949) and Speert (1952), although some disagreement will be expressed with the criteria used by the latter author in a few instances. In all of these studies the patient had carcinoma of the endometrium, and examination of prior dilatation and curettage specimens showed a high incidence of hyperplasia. Hertig and Sommers found 32 specimens adequate for study, including 7 with normal tissue (3 proliferative and 4 secretory), 1 with slight adenomatous hyperplasia, and the rest with definite hyperplasia. It is interesting to note that a carcinogenic agent, namely, radiation, had been given to 20 of these 32 women and that, in 13 cases in which the data were complete, an average dose of 1270 mg. hrs. of intrauterine radium had been administered in the average of 7 years prior to the final diagnosis of carcinoma.

Speert (1952) presented the data on 16 cases of endometrial carcinoma in which prior curettage specimens were available. Hyperplastic lesions were stated to have been found in 13 cases. In at least 2 of these (Nos. 7 and 10) we do not agree. Three prior specimens were available for case No. 7 (FIGURES 15, 16, and 17). Figure 15 is labeled glandular hyperplasia with intraluminal tufts of epithelium. To us this seems unremarkably normal midsecretory epithelium. Figure 16 shows "papillary intraglandular growth." Actually, this is a most unusual section demonstrating beautifully the normal intratubal protrusion of glandular epithelium found to be present in 38 per cent of normal endometria (as demonstrated in serial sections by Hampson and Gerlis, 1954). Speert's figure 17 again is supposed to demonstrate papillary intraglandular growth. To us this is nothing more than normal fraying of luminal surfaces of the cells and intra-luminal projection of portions of the epithelium caused by the cellular hypertrophy of the mid to late secretory phase. Six years later the curettage specimen shown in figure 18 undoubtedly shows adenocarcinoma, but to us it has no relation to the previously photographed normal phenomena. In case No. 10 the prior biopsy (FIGURE 23) was reported by the original pathologists as secretory. We concur and believe that again the "papillary intraglandular proliferation" is merely the normal phenomenon described by Hampson and Gerlis.

TeLinde *et al.* (1953) also have reported a retrospective study (groups II and III of their publication). Their group II will not be considered here, as the prior biopsy showed a questionable lesion in each instance. In their group III they had 8 patients with carcinoma subjected to curettage 3 to 34 years prior to being operated for adenocarcinoma. Only 3 showed hyperplasia. The remaining 5 were normal.

The difficulty with all such retrospective studies is the necessity of expecting a high incidence of hyperplasia. After all, women without hyperplasia but with normal endometrial function (who subsequently develop cancer) would have had no reason to be subjected to curettage, while those who had had hyperplasia in the past, with abnormal uterine bleeding, would necessarily make up the majority of the group with prior tissue available for study. Indicative of this is the fact that the 32 patients in Hertig and Sommers' study were culled from a group of 500.

Incidentally, it should be stated that TeLinde *et al.* did not infer that hyperplasia was an etiological agent for endometrial carcinoma.

Ovarian Stromal Hyperplasia and Endometrial Carcinoma

Definition of "ovarian stromal hyperplasia". The main characteristic of this condition is described as the presence of "masses of dense, well vascularized stromal tissue in either the cortex or the medulla of the ovary with a tendency to form whorls." In addition, some of the cells may become large and polyhedral so that they resemble luteinized cells of the theca interna. Nuclei tend to be plump and round. Connective tissue (collagen fibers) is scant, and reticular fibers are more prominent. Also, small structures called cortical granulomas appear (Smith *et al.*, 1942; Woll *et al.*, 1948). These findings are supposedly in contradistinction to those in the normally senescent ovary, in which there is an increase in connective tissue (collagen fibers), shrinkage of nuclei, lack of luteinized thecal cells, and absence of "cortical granuloma."

Association with endometrial carcinoma. It should be noted that some workers have found an increased incidence of stromal hyperplasia with carcinomas other than endometrial (breast, for example, and even in nonendocrine responding organs), but the present discussion will be limited to the association of this condition with endometrial carcinoma. A high incidence of "ovarian stromal hyperplasia" in patients with carcinoma of the endometrium was first noted by Smith *et al.* in 1942. In 1948 Woll *et al.* noted its presence in 84 per cent of 331 patients with endometrial carcinoma, but in only 44 per cent of 307 autopsy noncarcinomatous controls. Novak and Mohler (1953) had similar findings in 54 patients with carcinoma and 48 autopsy controls. In addition, they noted a higher incidence of "hilar" (or interstitial) cells in the carcinoma patients than in the controls. In 1956 Schneider and Bechtal found stromal hyperplasia in 52.3 per cent of 44 carcinoma patients and 35 per cent in a similar number of autopsy controls. McKay (1957) has recently reviewed this subject.

The inference or suggestion from these findings, of course, is that the hyperplastic stromal cells produce estrogens that are in turn etiological agents in producing the endometrial carcinoma. It must be admitted also that a very tentative suggestion was made that the reverse might be true, that is, that the carcinoma indirectly caused the stromal hyperplasia (Woll *et al.*, 1948).

Our interest in this problem was stimulated by the fact that "controlled" controls were not used in these studies; that is, operatively obtained tissues from live patients were compared with "dead house" material in which some degree of autolysis no doubt would be present. Of more importance, however, was the question of whether the stress of dying might alter ovarian morphology.

Accordingly, we collected surgical material for the noncarcinomatous controls as well as for the carcinomatous patients (Roddick and Greene, 1957). Incidentally, these patients were of comparable age and had had no radiation therapy. Since the relative amount of fibrous tissue (collagen fibers) and reticular tissue should be of importance, Milligan trichrome (for collagen fibers) and the Wilder reticulum stain were used, as well as routine hematoxylin and eosin. After completion of our initial studies, reticulum stains were abandoned, since the findings in each instance were exactly the obverse of those with the trichrome stain (that is, the more collagen, the less reticulum, and vice versa). Detailed tabulations were made by each of us individually. These included the number of germinal epithelium inclusion cysts and cortical granulomas, as well as the presence of "thecosis," hilar cells, amount of collagen, percentage of round or oval nuclei versus spindle-shaped nuclei (results in this last category were not reproducible and were not included in the final tabulations), and the presence and degree of sclerosis of ovarian blood vessels (we theorized that an "active" ovary should have less sclerosis of vessels than an "inactive" ovary).

Tabulations of the results showed no difference in the number of hilar cells, cortical granulomata, germinal inclusion cysts, thecosis, or in the amount of collagen in the two groups. The presence, absence, or degree of blood vessel sclerosis was not included in the original publication. There was, however, no difference between the two groups. The presence or absence of "cortical granulomas" correlated much better with the presence or absence of germinal epithelium inclusion cysts than it did with "thecosis."

In conclusion we suggested that the difference in our results and those reported by others might have been due to the use of inappropriate (autopsy) controls by others. This suggestion was rightfully challenged, so we set about collecting a group of ovaries from age-matched autopsy controls. These were in turn studied and tabulated, hematoxylin and eosin and trichrome stains being used (Roddick and Greene, 1958b). It was found that these ovaries *did* differ from operative cancer and operative control specimens in that the incidence of cortical granulomas (significant at the 1 per cent level), hilar cells (significant at the 5 per cent level), and thecosis (not statistically significant) was less in the autopsy than in either of the operative specimen groups. The amount of collagen was more variable, but not significantly different in the three groups.

Since this latter manuscript was submitted for publication, Sommers and Meissner (1957) have compared the findings in *autopsied* cancer cases and *autopsied* control cases. Their findings agree with the original reports on this subject and disagree with ours. Seventy-three per cent of the cancer patients had "stromal hyperplasia," as compared to 36 per cent of the controls. We are unable to explain these discrepancies.

As is obvious, in presenting our data we have deliberately avoided saying hat this patient has "stromal hyperplasia" and that patient has not. We beieve that too little is known about the anatomic limits of the variability of the ovarian cortex at various ages and that we can make such a diagnosis (with only moderate reservation) in extreme cases only; even then we wonder if it is only a normal variant of the normally variable normal.

All of this leads, indirectly at least, to a consideration of just what evidence exists for the belief that this "entity" produces or causes the production of estrogens. Unfortunately, no one (to our knowledge) has even correlated the morphology of the postmenopausal ovary and that of the endometrium. We have recently attempted to do this (Roddick and Greene, 1958a). In 63 of our original 82 cases (with and without carcinoma), uninvolved endometrium was available for study. The endometria were classified into atrophic (48), nactive hyperplasia (4), hyperplasia (7), and proliferative (4), and were crossndexed as to presence or absence of hilar cells, thecosis, cortical granuloma, and the relative amount of collagen present. No correlation could be found. The endometria were then further grouped according to those that were inactive (atrophic and inactive hyperplasia) and active (proliferative and hyperplastic) and were cross-indexed in a similar manner. Again, no correlations could be ound. Three of the patients with "active" endometria had been receiving estrogens, but even the exclusion of these three failed to produce correlation. From our data then, we feel free to conclude that within the limits of our study n the postmenopausal female there is no correlation between ovarian morphology and endometrial findings. There seems to us to be no evidence that postclimacteric ovaries produce estrogens, even when there is so-called ovarian tromal hyperplasia.

We hope that at least some of our readers will be left with the impression hat a frequently alleged relationship between endometrial carcinoma and esrogens should be given the Scotch verdict, "not proved."

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Discussion

Sheldon C. Sommers (*Massachusetts Memorial Hospitals, Boston, Mass.*): The researches described by H. Greene and Gardner represent landmarks in experimental pathology. Endometrial adenocarcinomas induced in rabbits by injections of long-acting estrogens showed somewhat different endocrine changes. Adrenocortical hyperplasia occurred, involving a different part of the zona fasciculata, and was often nodular, and there was a significant increase in anterior pituitary lobe cells termed hypertrophic amphophils.

Comparison of rabbits that developed cancer after estrogen with animals similarly treated that did not has suggested a two-stage carcinogenetic mechanism. In the first stage the injected estrogen stimulated the endometrium both directly and also indirectly via the pituitary-ovarian axis, with hyperplasia of pituitary gonadotropic basophil cells and ovarian cortical stroma. If no further endocrine abnormality developed, only endometrial hyperplasia was found. Some rabbits hyperreacted and subsequently developed a second endocrine abnormality comprising adrenocortical hyperplasia and increases of pituitary hypertrophic amphophil cells, coincident with the outgrowth of cancers. The hormones involved are as yet unknown.

Gardner's careful experiments indicate the carcinogenic importance of locally applied estrogen in carcinoma of the mouse cervix. In women dying with carcinoma of cervix there is no evidence of unusually frequent ovarian stromal hyperplasia or inferred excessive estrogen. Women with cervical carcinoma, like Gardner's mice, appear to be endocrinologically normal hosts exposed to some local carcinogen.

Grady provided a thoughtful review of the problems of trophoblastic tumors. The pathologist has the diagnostic duty of distinguishing between normal and neoplastic trophoblasts. The former shows no anaplasia, meaning abnormal variations in nuclear and cell sizes, shapes, staining, and arrangement. In hydatidiform mole the placental villi have lost their stromal vascularity. The trophoblast remains alive and, microscopically, may appear rather flamboyant, but lacks convincing anaplasia and invasion. In chorioadenoma destruens, aside from the gross nodule found in the myometrium, microscopic invasion is found. As Novak pointed out, intramuscular chorionic villi may be identified, which is not the case in the lethal choriocarcinoma.

In Ober's paper a very complete classification of uterine sarcomas is given. One pertinent case might be cited. After a pure rhabdomyosarcoma of the uterus had been removed 8 years previously, a patient died without residual cancer. Incidental findings included a fibromuscular hamartoma of the left kidney, a fibrovascular hamartoma of the spleen, and a large epidermal inclusion cyst of the umbilicus. This suggested some abnormal mixture of tissues early in embryonic life, supporting the dysontogenetic theory of some uterine sarcomas.

R. R. Greene's conclusions are not really unacceptable. Results naturally vary with the amount and type of one's material, and the manner of study. The general pathologist sees a number of autopsies of old women who have died of degenerative diseases, some of whom have ovarian cortical stromal hyperplasia. This means a nodular thickening of the superficial ovarian stroma, forming a peripheral layer at least 1 mm. thick. Ovarian stromal hyperplasia is accompanied ordinarily by evidence of vaginal estrogen effect, endometrial proliferation or a slight hyperplasia, an active appearance of mammary duct epithelium, and myoepithelium, with slight edema of adjacent intrinsic breast stroma, and bones with sturdy trabeculae. Old women with atrophic ovaries usually have atrophy of these structures as well.

Carcinoma of the endometrium appears, in its development, to be a multistage process, and estrogen seems most important approximately ten years before cancer outgrowth. If a woman has no endocrine abnormality other than ovarian cortical stromal hyperplasia and inferred estrogenic stimulation, only endometrial hyperplasia is likely to be found. Host reactions involving pituitary-adrenal abnormalities appear to be associated more closely with cancer.

Part IV. Blood Coagulation in Pregnancy, Labor, and the Puerperium

FIBRINOGENOPENIC ACCIDENT OF PREGNANCY AND DELIVERY: A SYNDROME WITH MULTIPLE ETIOLOGICAL MECHANISMS*

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A short outline of a working hypothesis of the hemostatic mechanism in man is pertinent to a discussion of the hemorrhagic states associated with obstetrical accidents, and will be useful for future reference in the course of this paper. The theory presented here is one that seems acceptable to most investigators. It applies specifically to the hemostatic mechanism following traumatic injury.

As its continuity is destroyed, the metarteriole vessel contracts through an axon reflex. Platelets, originally at the center of the blood stream, move to the periphery, thus coming in contact with the site of injury, where they agglutinate. Chemicals of various natures are released. Generalized vasoconstriction follows, and the process of blood coagulation begins (FIGURE 1) and proceeds in rapid steps. Tissue and plasma thromboplastin, thrombin, and fibrin are formed in succession through the interaction of many primary agents. Anticoagulant mechanisms and autocatalytic reactions are not discussed for reasons of space. An additional aspect of the coagulation process is the fibrinolytic mechanism; its possible role in physiological clotting remains a moot question. It is believed that intravascular clotting occurs at all times within the circulation, and that continuous fibrinolysis only prevents the catastrophe of total thrombosis.

It is thought that, at least in part, the hemorrhagic states of pregnancy and delivery are often referable to a state of hypercoagulability and of intravascular clotting. This is a special instance of intravascular coagulation, different from the more familiar process of arterial occlusion, of phlebothrombosis, and of thrombophlebitis, where lesions of the vascular wall are of primary importance. In the obstetrical hemorrhagic state there is no lesion of the vascular wall. Rather, the mechanism for intravascular coagulation lies within the blood vessel itself, possibly an exaggeration of mechanisms leading to formation of the clots in physiological conditions. Many changes occur in factors and mechanisms of blood clotting in the pregnant woman that may lead to hypercoagulability. These include hyperprothrombinemia, hyperfibrinogenemia, and the increased ability of the serum to accelerate the clotting of normal plasma (so-called convertin activity). Clinically, these findings may be correlated with the common occurrence of thrombosis during pregnancy. The changes de-

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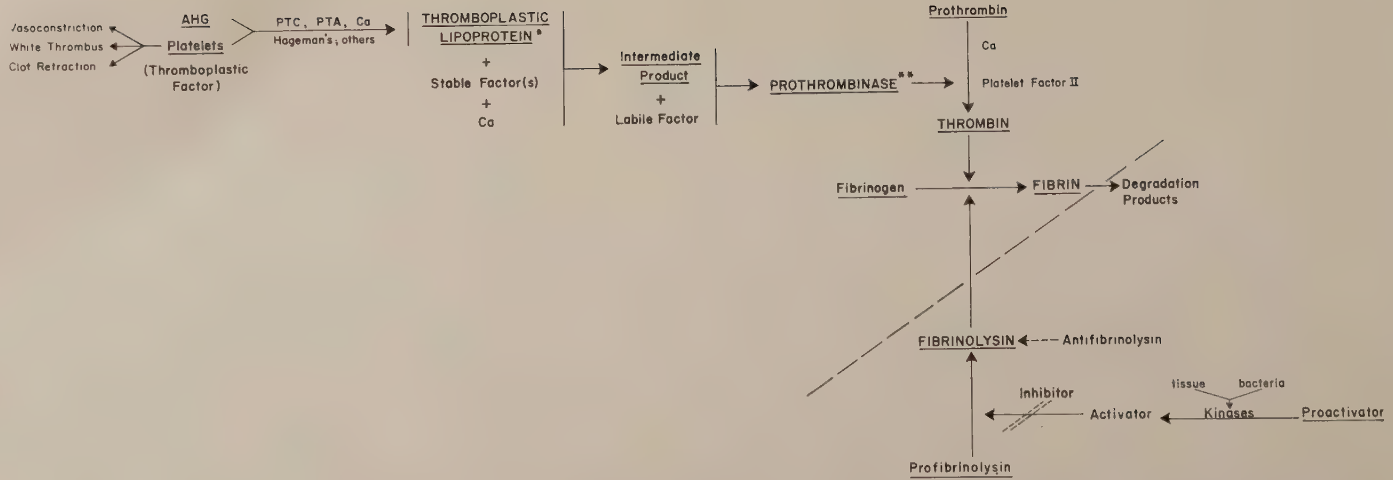

FIGURE 1. A summary of the hemostatic mechanism in man following trauma. Platelets are responsible primarily for vasoconstriction and clot retraction. Their agglutination which supplies a hemostatic plug (white thrombus), also releases a thromboplastic factor. This combines with antihemophilic globulin (AHG) to form a substance (thromboplastin lipoprotein) with activity of tissue thromboplastin. Other agents—plasma thromboplastic component (PTC), (?) plasma thromboplastin antecedent (PTA); calcium; Hageman, Stuart, Carr, and Prower's factors—some real, some hypothetical—are needed for PTC or simply accelerate this phase. The thromboplastic lipoprotein reacts with the stable factor (or factor VII) and calcium to form an intermediate substance which, in turn, combines with the labile factor (factor V) to produce prothrombinase, or plasma thromboplastin. This easily precipitable factor, possibly an enzyme, converts prothrombin to thrombin in the presence of calcium and a hypothetical platelet factor (II). As thrombin is formed, it converts fibrinogen to fibrin.

Anticoagulants that antagonize each factor and each phase of the clotting process are present. Of special interest to this discussion is an antithromboplastin inhibiting the thrombo plastic lipoprotein activity.

The fibrinolytic mechanism consists of a series of phases. From plasma proactivator, tissue kinases form an activator that, in turn, converts profibrinolysin or plasminogen (an inactive proenzyme) to fibrinolysin or plasmin. There is an inhibitor of the activator and of the active enzyme (antifibrinolysin or antiplasmin). As in animals, it is possible that tissue kinase may activate fibrinolysin directly from profibrinolysin. Bacterial kinases also activate fibrinolysin, but they appear to need the alternate way of the proactivator → activator system.

scribed regress promptly at termination of pregnancy. In fact, during delivery there is a temporary but appreciable exaggeration of clot lysis. Abnormalities are more pronounced and of more complex nature if pregnancy is abnormal. However, while "hypercoagulability" is common, obstetrical fibrinogenopenic states are uncommon. Thus, additional mechanisms to determine their occurrence must exist. A few factors related to the presence of placental, amniotic, and fetal constituents in the uterine cavity, suggest themselves. We must consider entrance into the circulation of materials from the uterine wall, the placenta, the amniotic sac; the role of anaphylactic and allergic phenomena due to the presence of fetal materials; and, finally, the possible role of hemorrhage. Here we shall report on a series of experiments designed to evaluate the role of each mechanism in the pathogenesis of "fibrinogenopenia" and shall attempt to correlate such findings with our clinical observations.

Experimental Studies

Infusion of uterine tissue (*Figure 2*). Saline extracts of homologous muscle and endometrial lining exhibited fair tissue thromboplastic activity and no fibrinokinase activity.* When injected intravenously in dogs, they caused a significant but transitory drop in platelets and in plasma fibrinogen level.

Infusion of amniotic fluid in untreated and "sensitized" dogs. An analytical study of the clotting properties of human amniotic fluid (AF) has been carried out in this laboratory.† Many of our results were in close agreement with those reported by others.[1] Amniotic fluid contained no primary clotting factors; it exhibited, however, tissue thromboplastic and fibrinolytic activity (TABLE 1) and antithrombin activity (TABLE 2). The thromboplastic activity, at least, appeared related to the corpusculate content, since the ability of AF to accelerate thrombin generation was directly proportional to the speed at which it had been centrifuged prior to use (FIGURE 3). AF contained proactivator and profibrinolysin, as shown in the fibrin plate heated at 85° C. (FIGURE 4), but no fibrinokinase. It was also a potential source of antifibrinolysin (TABLE 3). The latter also appeared related to the corpusculate content of AF.

The intravenous injection of canine or human AF in dogs caused changes in the coagulation mechanism, which depended, to a great extent, on the volume and the degree of particulation and of concentration of fluid. The rapid injection of 110 ml. of amniotic fluid in a 25-lb. dog was followed by a transient but sharp decrease in the number of platelets and in the plasma fibrinogen level. Changes of systemic blood pressure were minor (FIGURE 5). Amniotic squamae and fibrin deposits were detected in the large pulmonary vessels of dogs sacrificed 6 hours after the infusion. Findings were insignificant when smaller volumes were injected. Also, the administration of 70 ml. of human AF at aliquots of 10 ml. in the space of 4 hours induced only minor changes in

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* Uterine tissue contains high fibrinokinase activity, which, however, is located intracellularly in the mitochondrial fraction and, thus, is not soluble.

† In cooperation with Leo Gagliardi of Vercelli, Italy; formerly Research Fellow in Hematology, Joseph Stanton Memorial Laboratories of Saint Elizabeth's Hospital, Boston, Mass.

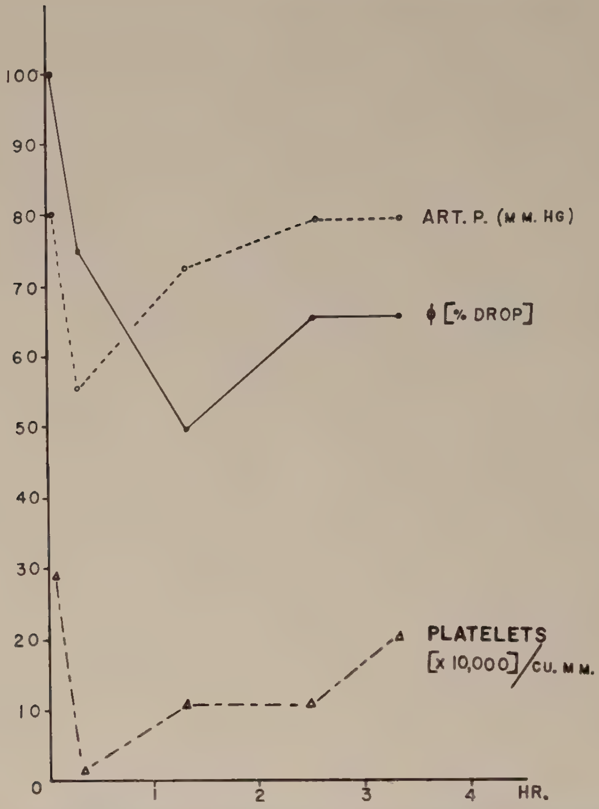

FIGURE 2. Effects of the intravenous administration of uterine extract on the hemostatic mechanism in dogs. The preparation, in saline, was able to give a prothrombin time of 30 sec. when used as the source of thromboplastin in a Quick 1-stage technique. Fifty to 100 ml. were administered intravenously by rapid infusion.

TABLE 1

CLOTTING ACTIVITY OF AMNIOTIC FLUID

AHG activity	0
PTC activity	trace
Platelet thromboplastic factor	0
Tissue thromboplastin activity	(= 150 γ rabbit brain tbp./100 ml.)
Prothrombin	0
Labile factor (factor V) activity	0
Stable factor (factor VII) activity	0
Fibrinogen	0
Thrombinlike activity	0
Antithromboplastin activity	0
Antithrombin activity	+
Profibrinolysin	+
Proactivator	+
SK-like activity	0
Antifibrinolysin	+

TABLE 2
ANTITHROMBIN ACTIVITY OF AMNIOTIC FLUID

Sample	Thrombin* + saline, sec.†	Thrombin* + amniotic fluid, sec.‡
1	90	110
2	100	100
3	110	300
4	120	300
5	120	240
6	140	220

Amniotic fluid and thrombin incubated at room temperature for 15 min. The mixture was added to 0.6 ml. of fresh human citrated plasma. Similar study with human brain and rabbit brain tbp. showed no antithromboplastin activity of amniotic fluid.

* Bovine, 0.5 μ/ml

† 0.3 ml.

‡ 0.3 ml., native.

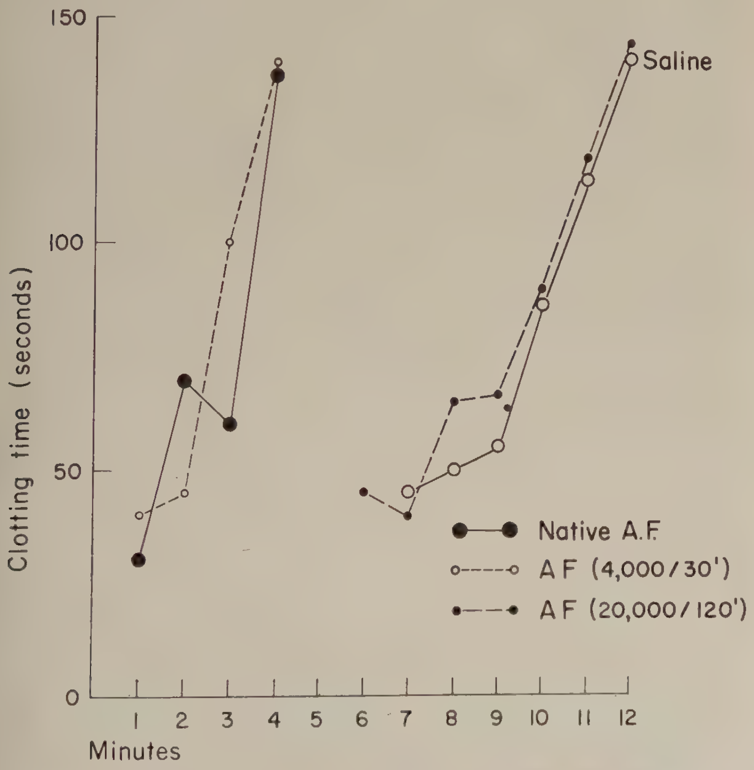

FIGURE 3. Effect of amniotic fluid on thrombin generation. One fifth ml. of amniotic luid (centrifuged at various speeds) or saline was added to plasma prior to recalcification. Clotting time became faster (faster generation of thrombin) when amniotic fluid was added o the reacting mixture, which had been centrifuged at slow speed.

the plasma fibrinogen level. An additional study was carried out, since it i conceivable that AF may penetrate into the material circulation during pregnancy (especially pathological pregnancy) and thus, perhaps, induce a state o sensitization. A dog received intravenously 15 ml. of homologous AF withou appreciable effect. Another 15-ml. aliquot was kept in frozen storage and administered 1 month later. Such volume, of no effect in untreated animals caused hypotension, fibrinogenopenia, and thrombocytopenia of several hours duration (FIGURE 6). The animal had possibly become sensitized to AF an thus responded to a minor exposure with severe fibrinogenopenia. Since AF i primarily of fetal production, mechanisms of sensitization are theoretically important in man.

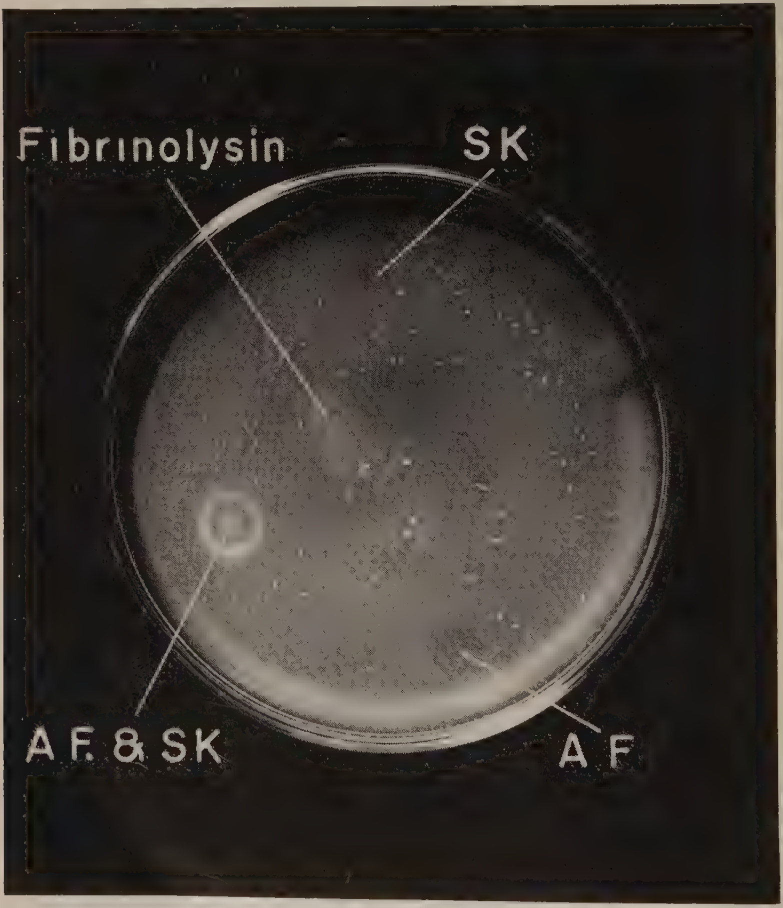

FIGURE 4. Seeding of a fibrin plate heated at 85°C. with various reagents and with amniotic fluid. Note (1) the large area of lysis with fibrinolysin (Loomis), (2) the absence o lysis after seeding with AF and with SK (streptokinase-streptodornase), and (3) the lysis of th plate after seeding with AF and SK (amniotic fluid incubated with streptokinase-streptodornase). AF probably contains profibrinolysin and proactivator; there is no kinase activity.

TABLE 3

AMNIOTIC FLUID AS A SOURCE OF ANTIFIBRINOLYSIN

omis fibrinolysin*			0.2 ml.		
tive amniotic fluid (0.2 ml.), dilution:	1	½	¼	1/10	0
	Incubation at 37°C. for 30 min.				
rinogen†			0.3 ml.		
rombin‡			0.1 ml.		
ysin time	18 min.	15 min.	12 min.	9 min. 30 sec.	8 min.

Amniotic fluid inhibits activity of fibrinolysin (activity is no longer present after cen-
ugation of amniotic fluid at 20,000 rpm/120 min.).

* Five mg./ml.

† Bovine, 300 mg./100 ml.

‡ Bovine, 100 NIH units/ml.

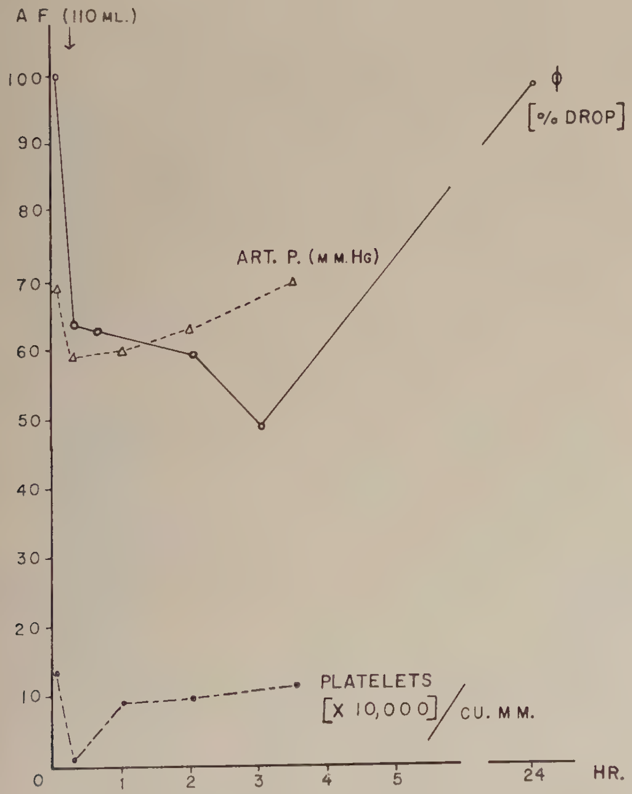

FIGURE 5. Rapid infusion of a large volume of human amniotic fluid in the dog. Note
sharp, transient fall in fibrinogen and platelets following the rapid, intravenous injection
a large volume of AF in an untreated dog.

Infusion of placental extracts. Used in the experiments was a diluted salin extract of human placenta giving a prothrombin time of approximately 20 se when used as a source of thromboplastin in a Quick 1-stage system. The e tract exhibited no fibrinokinase activity in *in vitro* tests. The administratio of such material to rabbits was followed by profound shock and by a majo breakdown of the hemostatic mechanism. Two phases were observed. In a early phase, lasting up to 60 min., there was some elevation in the number platelets. Fibrinogen level, however, fell almost immediately. Changes in a tivity of prothrombin and labile factor were less significant (FIGURE 7). Th thromboplastin generation test, using normal platelet substrate and the ani mal's plasma and serum, showed increased (but not faster) production of plasm thromboplastin. Tests were repeated using only serum or plasma from th experimental animal, the alternate reagent being from normal animals. Bot

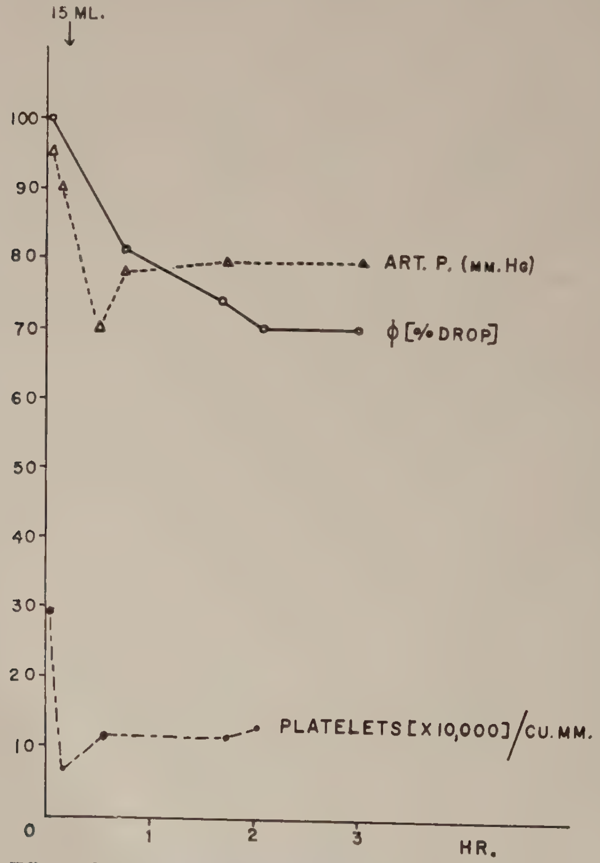

FIGURE 6. Effects of repeated injection of homologous amniotic fluid in a sensitized dog The dog received intravenously 15 ml. of amniotic fluid without effect. The injection of 1 ml. 1 month later was followed by hypotension, sustained fibrinogenopenia, and thrombocyto penia.

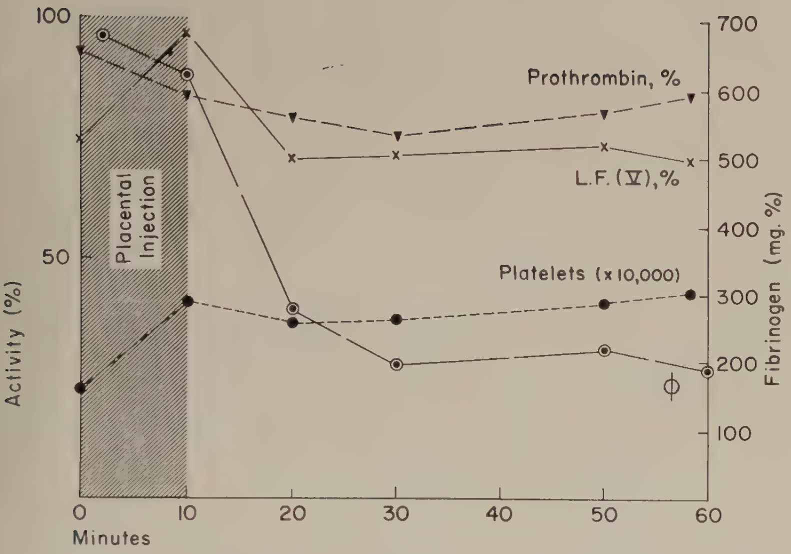

FIGURE 7. Placental infusion in the rabbit: early response. Placental thromboplastin was diluted until it gave a prothrombin time of approximately 20 sec. in a Quick 1-stage method. Three ml. was injected intravenously in 20 to 30 min. Note the sustained elevation in the number of platelets, as compared to the severe drop in plasma fibrinogen level.

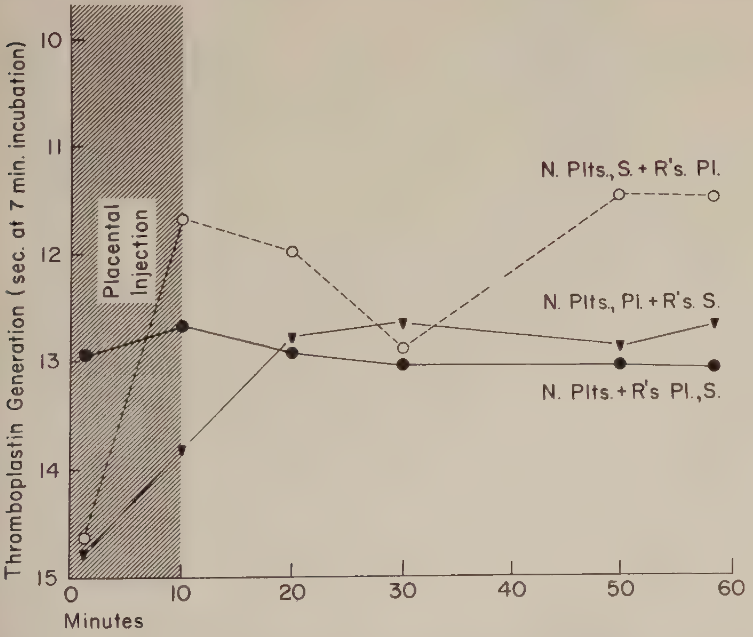

FIGURE 8. Effect of the placental infusion on the generation of thromboplastin in rabbits: early phase. The test was performed after 7 min. of incubation of reagents. Materials from normal or experimental animals were used as indicated. The results suggested the appearance of clot-accelerating factors in the plasma and serum after infusion. Legend: N. Plts. = human platelets; R's. Pl. = plasma from infused rabbit; N. Plts., Pl. = human platelets—plasma from untreated rabbit; R's. S. = serum from infused rabbit; R's. Pl., S. = plasma and serum from infused rabbit. Human platelets can be substituted for rabbit platelets without affecting the results (R.T.).

plasma and serum appeared to contain factors that increased the yield but no the speed of generation of thromboplastin (FIGURE 8). Fibrinolytic activity o serum rose immediately. Antifibrinolytic activity also increased, although les so (FIGURE 9a). The late phase lasted approximately 24 hours. Fibrinoge platelets, and labile factor were very low (FIGURE 10). Thromboplastin ge eration appeared poor also (FIGURE 11). There was sustained elevation o fibrinolytic activity of serum, while the behavior of antifibrinolysin was errati (FIGURE 9b). The appearance of an anticoagulant inhibiting the activity o tissue thromboplastin was typical of this phase (FIGURE 12). Hemolysis wa visible with the naked eye during both the early and late phase. Red-ce mechanical fragility was increased also. A phase of recovery followed, all va ues returning slowly to normal in 72 hours, with the exception of the anticoagu lant, which was still present several days later.

Animals were sacrificed 1 to 2 hours after the injection of placental extrac At macroscopic examination, premortem thrombi were found primarily in th

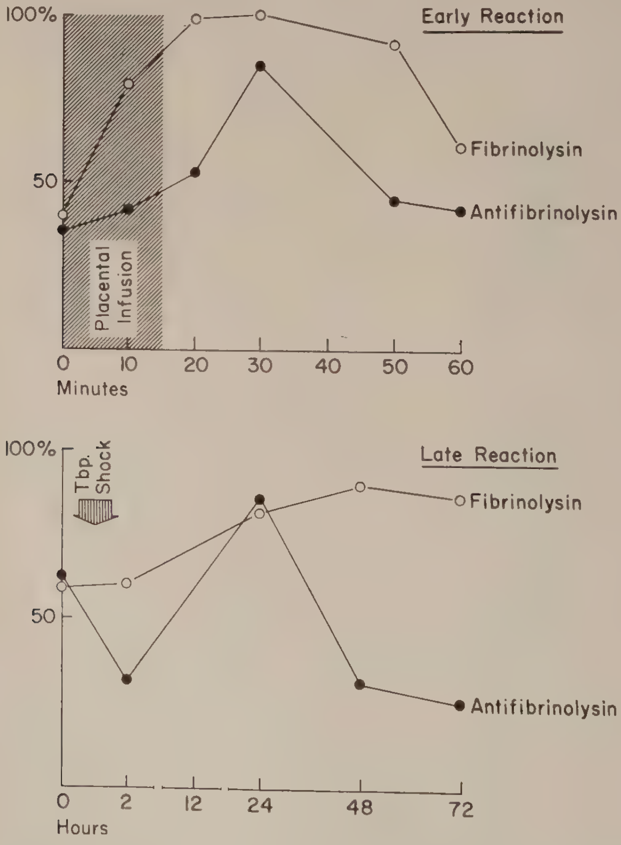

FIGURE 9. Fibrinolytic system following placental infusion; plate method.

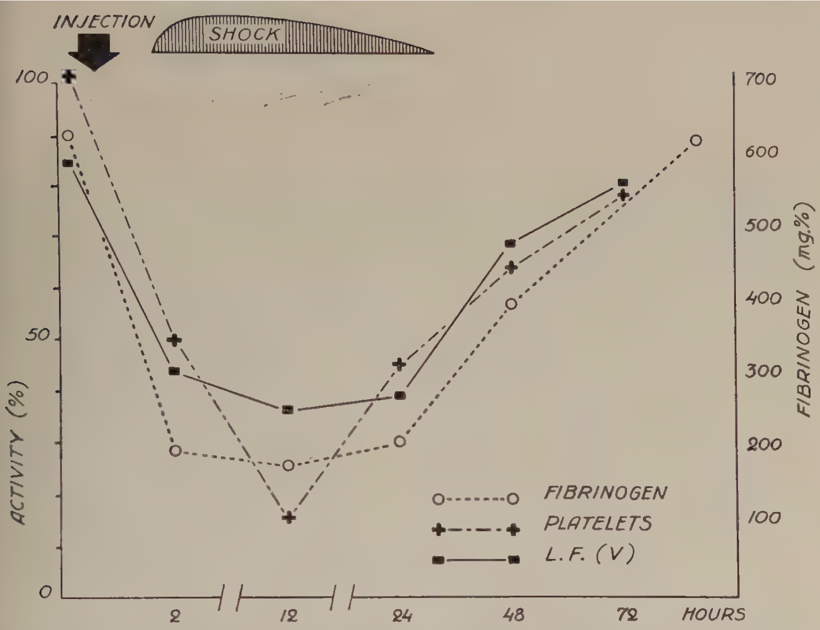

FIGURE 10. Injection of placental extract in rabbits: late and recovery phase. Note the depletion of platelets, fibrinogen, and labile factor. Maximum response was at 12 hours and was followed by slow return to normal value, complete in 72 hours.

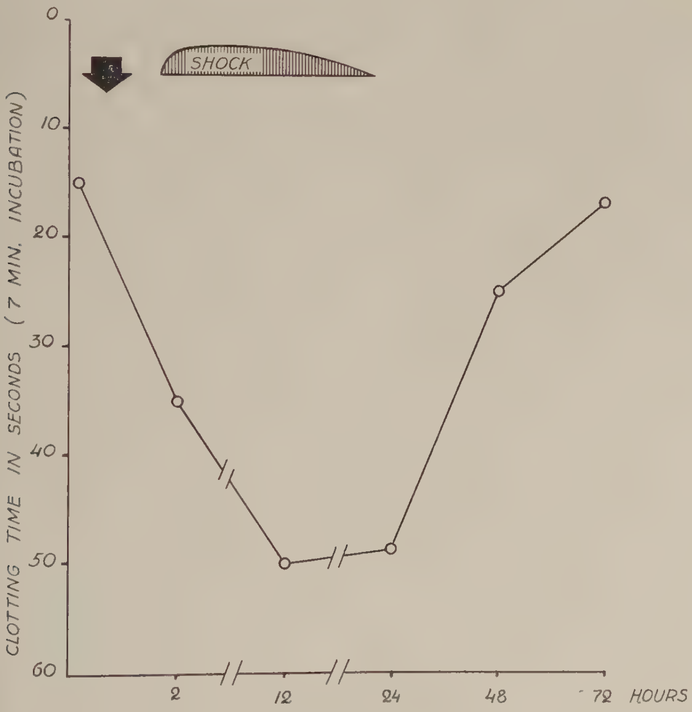

FIGURE 11. Placental infusion in the rabbit: late phase. Behavior of the thromboplastin generation test.

major pulmonary vessels, causing multiple pulmonary infarcts (FIGURE 13 Microscopic studies showed edema of the alveolar walls and filling of the entire pulmonary vascular tree with material of the appearance and staining reactions of fibrin. Alveoli were filled by an exudate containing erythrocytes No lesions were found in animals sacrificed 96 hours after placental infusion.

Infusion of tissue fibrinokinase. A homogenate of lung tissue was centrifuge as described by Lewis and Ferguson[2] to obtain a mitochondrial fraction wit fibrinokinase activity. The fraction had good fibrinokinase, but also moderat

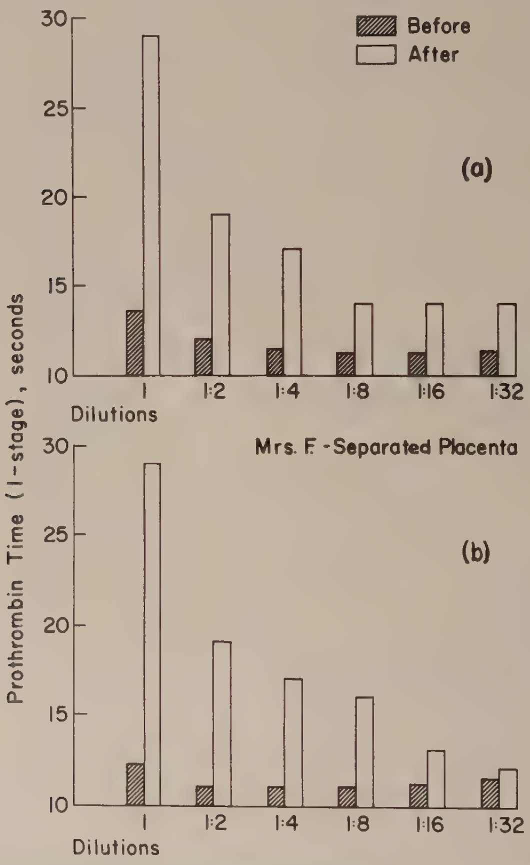

FIGURE 12. Circulating antithromboplastin anticoagulant: (a) in rabbits after infusio of placental thromboplastin; (b) in a woman with separation of placenta and "fibrinogen penic" syndrome. The columns indicate the inhibition of tissue thromboplastin activity b varying dilutions of serum. Controls (shaded columns) were the rabbit's serum prior to th study, and normal human serum (woman's) prior to the accident.

Technique: (1) the serum to be tested was diluted by the method of progressive dilutio with Michaelis' buffer modified by Owren-Koller (barbital-saline) buffer; (2) each dilution wa incubated with placental thromboplastin for 30 min. at 37°C. at equal volumes; (3) th incubated thromoboplastin-serum mixture was used as the source of thromboplastin in Quick's 1-stage procedure, using standard oxalated plasma.

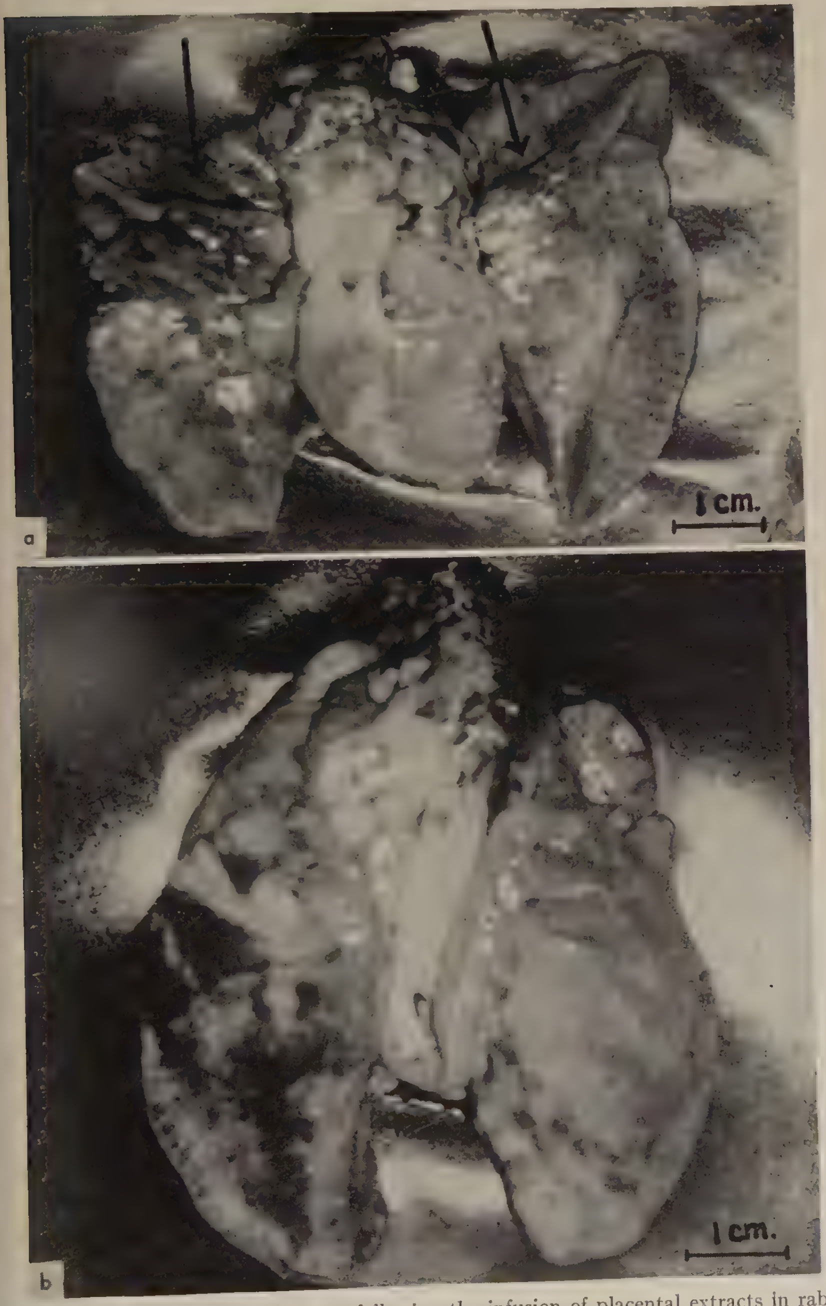

FIGURE 13. Macroscopic findings following the infusion of placental extracts in rabbit: (a) occluded pulmonary vessels, with areas of lung infarction: (b) pulmonary infarcts.

thromboplastic activity. Rabbits received 1 mg. of fraction/kilo weight intravenously. The results were equivocal, possibly because of the simultaneou presence of fibrinokinase and thromboplastin activity.

Activity of prothrombin and labile factor (V) fell moderately, and promptl returned to normal within 24 hours. Plasma fibrinogen level fell to abou half the original value within 2 hours of injection. Platelet count was lowes 12 hours after the injection. Both platelets and fibrinogen were back to norma within 48 hours (FIGURE 14a). There was decreased generation of thromboplastin, most evident 12 to 24 hours after the injection (FIGURE 14b). Fibrino

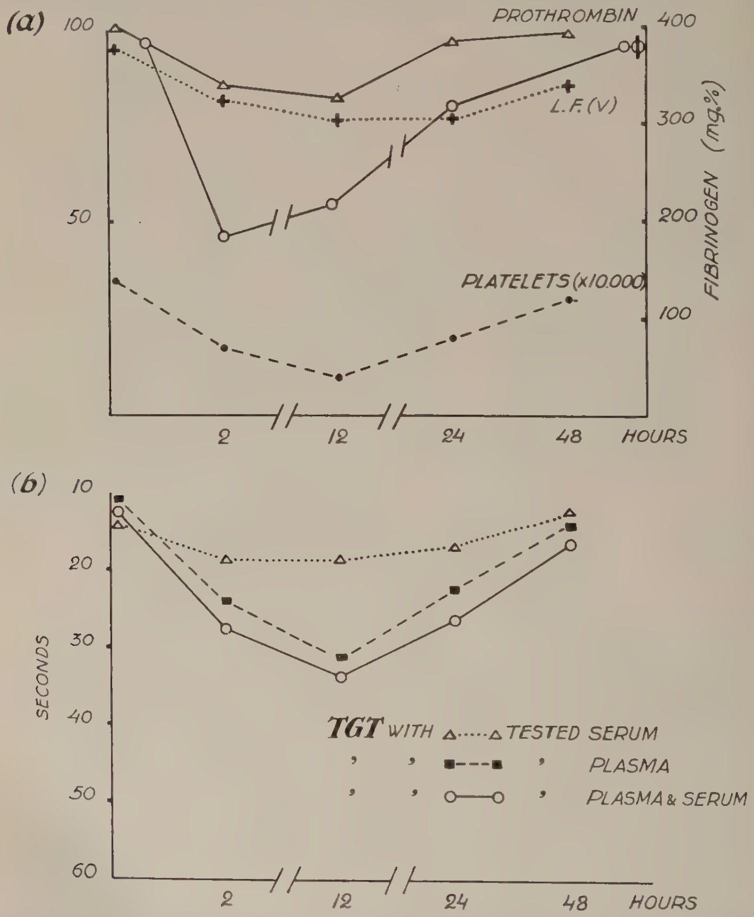

FIGURE 14. Injection of tissue fibrinokinase in rabbits: 1mg. of tissue fraction VI (Claude homogenized in saline was injected intravenously at slow speed (15 min.). (a) Behavior o platelet count, plasma fibrinogen level, activity of prothrombin, and labile factor (V); (b thromboplastin generation test. Normal human platelets were used in each case; plasma an serum from experimental animals (TESTED) as well as from normal were used as indicated As compared to the infusion of thromboplastin, there was no development of clot acceleratio in plasma or serum.

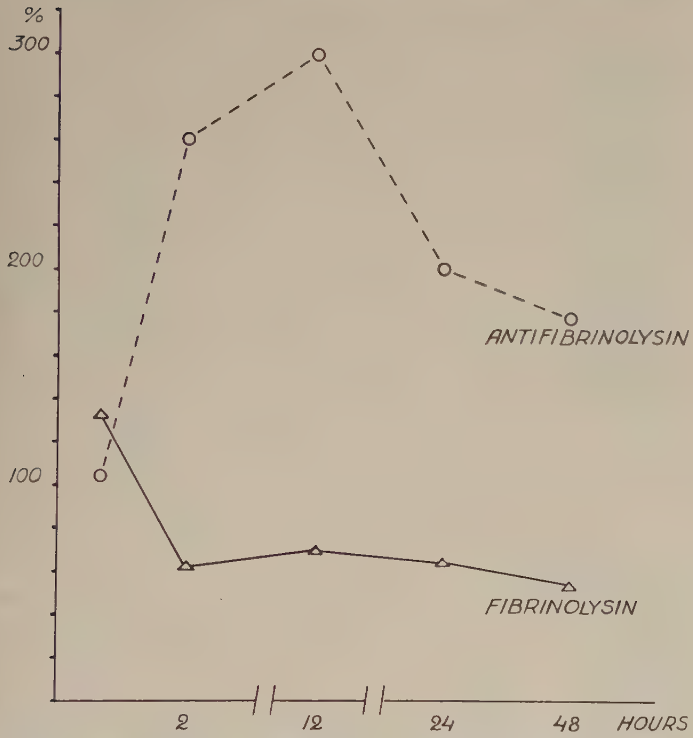

FIGURE 15. Fibrinolysis and antifibrinolysis following infusion of tissue fibrinokinase.

ytic activity showed a very short-lived rise (not shown in FIGURE 15), followed
y a slight fall. There was a spectacular increase in antifibrinolytic activity
f serum, which had not returned to normal within 48 hours.

Hemostatic abnormalities in hemorrhagic shock. Earlier work on dogs* indi-
ated that severe bleeding may lead to significant fibrinogenopenia. Accord-
ngly, three fourths of the calculated blood volume was removed rapidly from
lbino rabbits through a needle inserted in the central artery of the ear. Shock
eveloped. Samples of blood were removed at various intervals of time. The
ndings emphasized the existence of an early and a late phase of reaction, as
een after thromboplastin infusion. In an early phase (40 to 60 min.) the
umber of platelets rose, while plasma fibrinogen level fell markedly. Activity
f labile factor (V) and complement titer also decreased, even after cessation of
leeding (FIGURE 16). There was higher yield of thromboplastin in the genera-
on test, possibly due to the presence of clot-promoting agents in plasma and
erum (FIGURE 17). The fibrinolysin decreased, while antifibrinolysin rose (FIG-
RE 18a). During a late phase (lasting up to 24 hours) there was a continued
ll in platelets, fibrinogen, and labile factor (FIGURE 19), most evident at 12

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* In cooperation with Hector Marin and Leo Gagliardi.

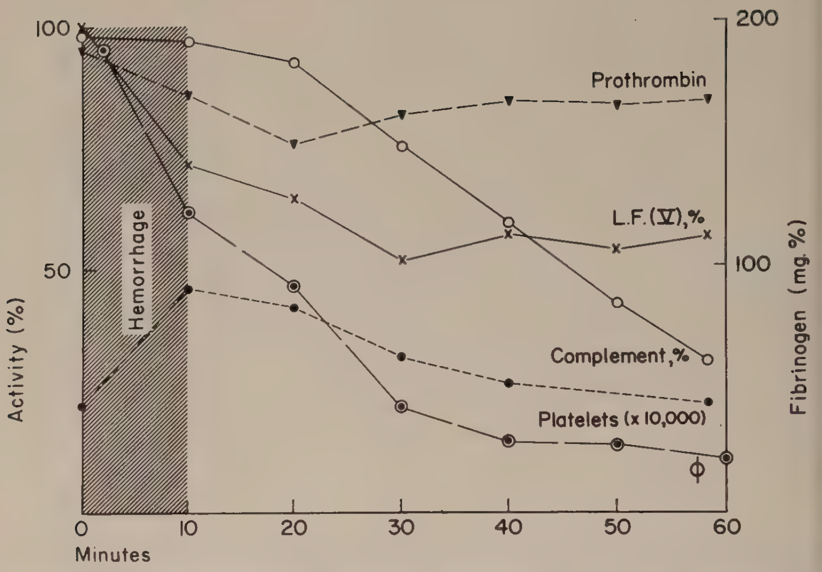

FIGURE 16. Hemorrhagic shock (produced by removing 57.5 ml. blood/kilo weight in 1 to 15 min.) in rabbits: early response. Platelets, various clotting factors, and complement

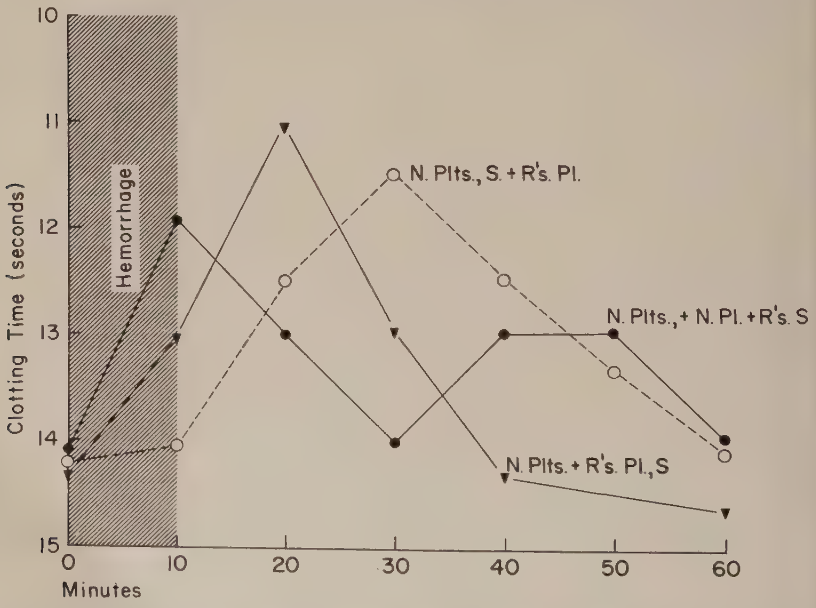

FIGURE 17. Hemorrhagic shock in rabbits: early response. Thromboplastin generation. The graph shows the clotting times obtained after 7 min. of incubation of the various reagents. Plasma and serum were collected from the animals under study at various intervals of time after bleeding, and from untreated animals. Normal and abnormal reagents were variously combined. Experiments suggested the presence of clot accelerators in plasma and serum. Legend: N. Plts., S + R's. Pl. = normal platelets and serum, tested rabbit's plasma; N. Plts. Pl. + R's. S. = normal platelets and plasma, tested rabbit's serum; N. Plts., + R's. Pl. S. = normal platelets, tested rabbit's plasma and serum.

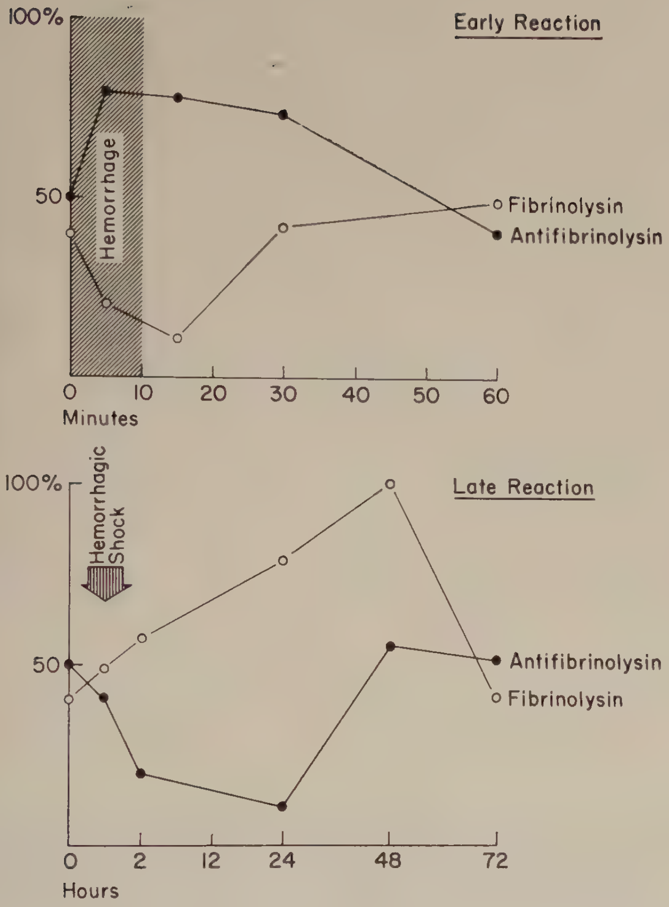

FIGURE 18 Hemorrhagic shock: fibrinolysin and antifibrinolysin.

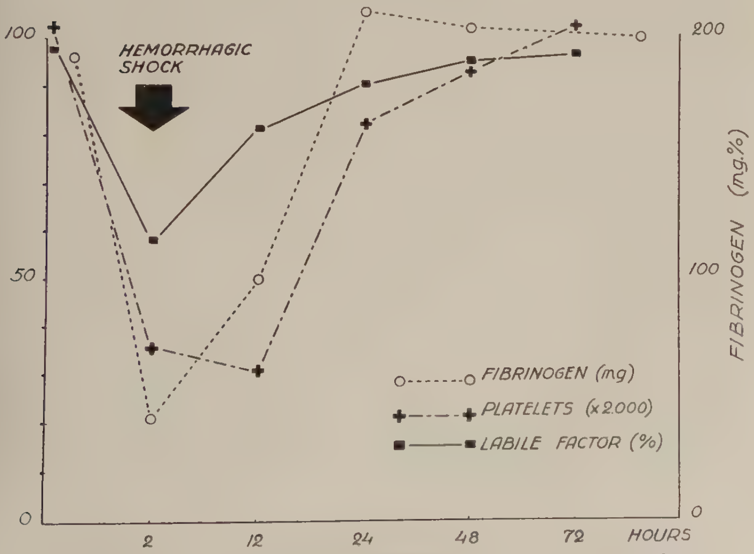

FIGURE 19. Hemorrhagic shock: late phase. Behavior of clotting factors.

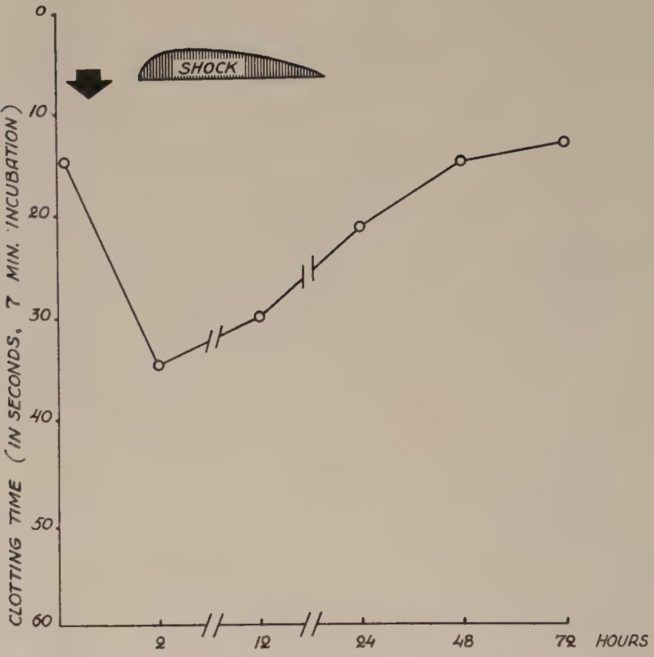

FIGURE 20. Hemorrhagic shock: late phase. Generation of thromboplastin.

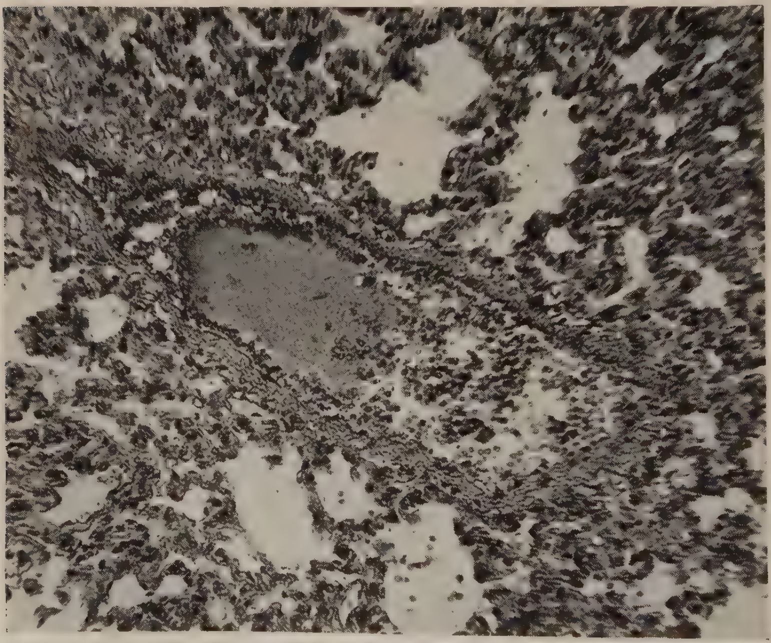

FIGURE 21. Hemorrhagic shock: microscopic findings in the lung. Note the intravascular deposition of fibrin. The animal was sacrificed 2 hours after hemorrhagic shock. ×24

hours. The fibrinolytic activity rose, while the antifibrinolytic activity fell (FIGURE 18b). Generation of thromboplastin became poor and remained so for several hours (FIGURE 20). The recovery phase lasted up to 72 hours. Neither anticoagulants nor hemolysis was detected at any time.

Macroscopic examination of animals sacrificed at various times was negative. Microscopic examination, on the other hand, revealed deposits with the appearance of clots of fibrin within the large vessel of the lung and liver (FIGURE 21). These were no longer evident in animals sacrificed after 48 and 96 hours.

Summary of Experimental Findings

TABLE 4 shows, in schematic form, a summary of our findings. Insufficient data were available to evaluate fully the effects of the infusion of saline extracts from uterus and of amniotic fluid. It could be expected, however, on the basis of *in vitro* testing, that these effects probably would have been similar to those obtained after placental infusion, although less pronounced. Behavior of clotting agents following infusion of tissue fibrinokinase, administration of placental thromboplastin, and production of hemorrhagic shock were quite similar in the late phases, although varying in intensity. Early acceleration of clotting and an early elevation of platelets were common to thromboplastic and hemorrhagic shock. The appearance of a tissue antithromboplastin coagulant and of hemolysis were typical of thromboplastic shock, possibly because of its greater intensity.

An analysis of the behavior of the fibrinolytic system requires a few comments on the techniques used in these experiments. Antifibrinolysin was expressed as the ability of the serum to inhibit the lysis of the fibrin plate by purified profibrinolysin activated with streptokinase. Fibrinolysis was measured on the heated plate, because serum did not lend itself to reliable studies with synthetic substrates, at least not in our experience. Since fibrinolytic activity

TABLE 4

FINDINGS IN SEVERAL EXPERIMENTAL FIBRINOGENOPENIC STATES

Infusion of:	Platelets	Fibrinogen	TGT	Clot accelerators	Fibrinolytic activity	Antifibrinolytic activity	Hemolysis	Anticoagulant
Uterus	−	−	−					
Amniotic fluid								
single (massive)	−	−	−					
in "sensitized" animal (small)	−	−	−					
Placenta:								
early phase	+	−	+	+	+	+	+	0
late phase	−	−	−	0	+	−	+	+
Fibrinokinase (tissue)	−	−	−	0	−	+	0	±
Hemorrhagic shock:								
early phase	+	−	+	+	−	+	0	0
late phase	−	−	−	0	+	−	0	0

Legend: − = decreased; + = increased; 0 = no variation.

of serum was evaluated after full activation with streptokinase, the entit measured was a combination of activated fibrinolysin originally present and o the remaining profibrinolysin. Thus, it was difficult to establish whether a low fibrinolytic activity meant its absence or, rather, the consequence of massiv activation *in vivo* with little profibrinolysin remaining to be consequently activated *in vitro*. We relied to a certain extent upon the behavior of antifibrinolysin, which probably increases in response to exalted fibrinolytic activity *i vivo*. In the late phases of the shocks studied, there was a striking dissociation between fibrinokinasic shock (showing low fibrinolytic and high antifibrinolyti activity) and placental and hemorrhagic shock (showing high fibrinolytic an low antifibrinolytic activity). Massive activation of fibrinolysin probably occurred while, in the other instances, the activation was probably less intense There was also an additional interesting dissociation in the early phases of th placental and hemorrhagic shocks. In placental shock, high fibrinolytic an antifibrinolytic activities were present, indicating perhaps an early, but no massive, activation of fibrinolysin. In the early phase of hemorrhagic shock on the other hand, there was low fibrinolytic and high antifibrinolytic activity This behavior was extremely difficult to interpret, unless it means activatio of fibrinolysis earlier than in placental shock.

Table 4, brings out the great difficulty in distinguishing the nature of th phenomena observed on the basis of laboratory findings alone. It also emphasizes the great importance of the time during which these reactions are observed It would appear that, in all experiments carried out, intravascular clotting an activation of fibrinolysin were closely associated although, perhaps, differently timed. Very surprising and perhaps significant were the close similarities between thromboplastic (placental) and hemorrhagic shock.

Clinical Observations

Fibrinogenopenia is an increasingly common finding in many acquired hemorrhagic syndromes. It is perhaps most common in four obstetrical complications: (1) separation of placenta;[3, 4] (2) amniotic embolism;[5] (3) intrauterine fetal deaths;[6] and (4) attempts at criminal abortion.[7] Other clinical syndromes encountered during pregnancy and delivery may be accompanied by fibrinogenopenia, but they are extremely rare. In the course of the past ten years we have observed a number of cases of "fibrinogenopenia" during pregnancy and delivery. They included a number of cases where a definite diagnosis o separated placenta, amniotic embolism, or intrauterine death could be made We did not observe any cases due to criminal abortion. At the acme of bleeding, most of our cases presented fibrinogenopenia, thrombocytopenia, an changes in the fibrinolytic system. Great difficulty was experienced in evaluating the pathogenesis of the hemostatic breakdown in each case. Most of th accidents were so acute that it was impossible to delay therapy, thus alterin the clinical and laboratory picture. Most of the observations, in fact, include only laboratory studies at the time of acute hemorrhage. Another difficult consisted in the many technical limitations in the studies of clotting and fibrinolytic phenomena. Finally, it was hazardous to determine the exact mechanism of the accident whether, for example, separation of placenta was complicated by amniotic or placental infusion. Such information was available only in those cases where hysterectomy became imperative or where the patient died. Correlation of clinical, laboratory, and anatomical findings was thus very limited.

As stated previously, multiple mechanisms may be able to cause the classic picture of thrombocytopenia and fibrinogenopenia, as it is observed in the

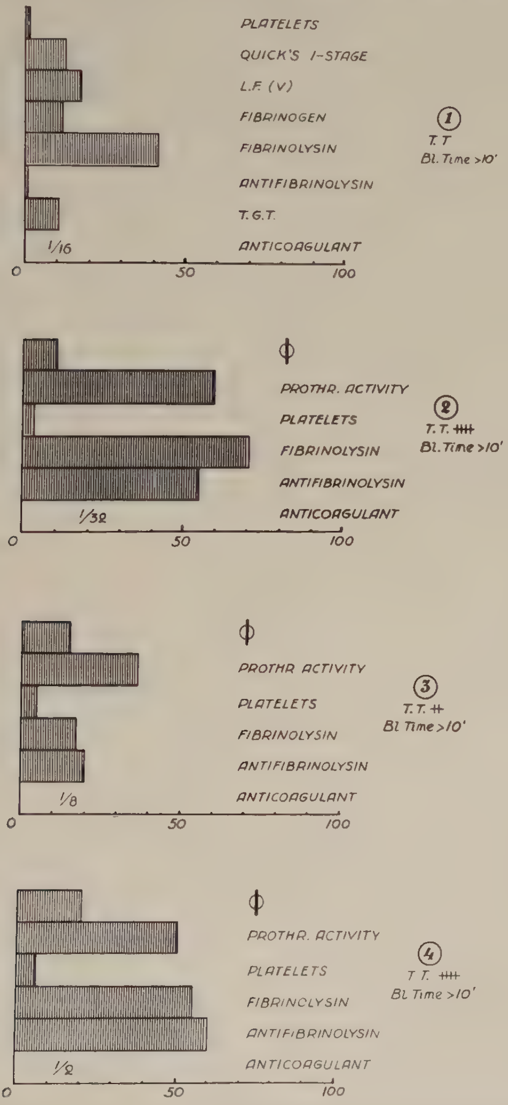

FIGURE 22. Findings in 4 instances of separation of the placenta with hemorrhagic endency.

hemorrhagic accidents of pregnancy. Experiments as different as injection o thromboplastic material, injection of tissue kinases, and hemorrhagic shoc were equally able to produce a picture simulating that found in obstetrica fibrinogenopenia. Also, in our clinical experience, laboratory findings were no uniform, even when histological studies revealed a similar pathogenetic mechanism. FIGURE 22 shows findings in four cases of separation of placenta wher hysterectomy became necessary. The gross pathology was essentially similar Large thrombi were present in the uterine veins and, histologically, there wa massive deposition of fibrin within the vessels. Still, one patient showe fibrinolysis and a circulating anticoagulant in addition to fibrinogenopenia an thrombocytopenia (case 2). Another patient (case 3) showed severe fibrinogenopenia and thrombocytopenia, also with a circulating anticoagulant. Lyti phenomena were minor. A third patient (case 4) showed fibrinogenopenia thrombocytopenia, and some activation of fibrinolysin, but low anticoagulant

A similar problem was encountered in amniotic embolism with or withou separation of placenta (FIGURE 23). The cases considered were only thos proved by finding amniotic elements within the vessels of available organs The second patient in FIGURE 23 underwent hysterectomy, and sections showe filling of vessels with amniotic squamae. In this case, however, the presenc of a circulating anticoagulant and thrombocytopenia was predominant, whil

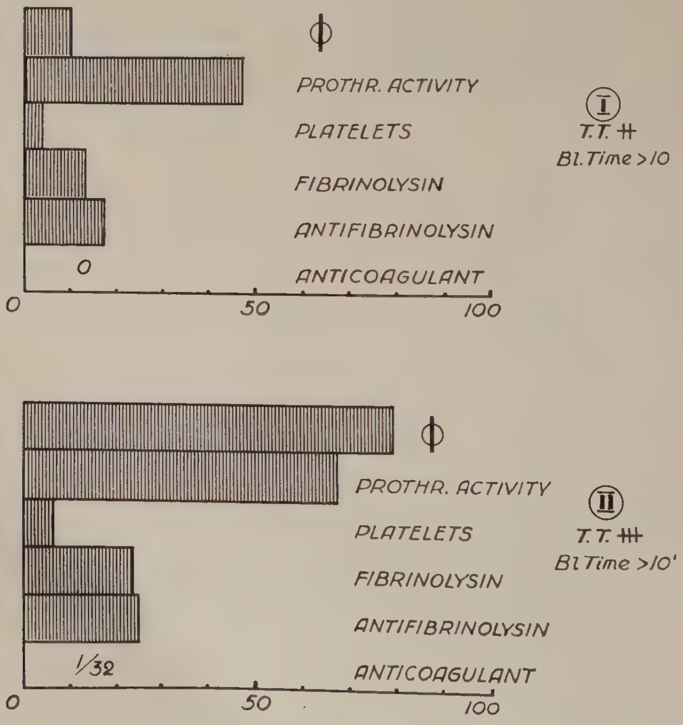

FIGURE 23. Findings in two instances of amniotic fluid embolism.

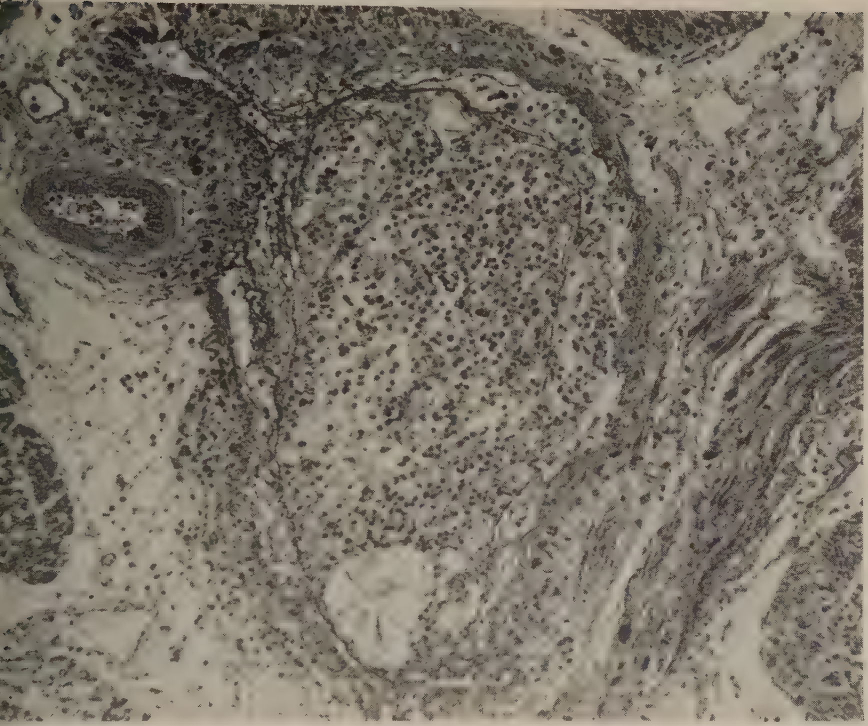

FIGURE 24. Uterine vessel filled with amniotic squamae and fibrin deposits engulfing blood cells.

ytic phenomena were minor and there was normal fibrinogen level. Such indings defy explanation. In another case, thrombocytopenia and fibrinogenopenia were outstanding. The patient came to autopsy. Again the uterine (FIGURE 24) and pulmonary vessels were filled with fibrin deposits and amniotic squamae.

The patients with hemorrhagic tendency following intrauterine death of the etus, on the other hand, showed fairly uniform findings consisting in severe nd persistent thrombocytopenia, fibrinogenopenia, and the appearance of an nticoagulant, of fibrinolysis, and of fibrinogenolysis. The anticoagulant, howver, was at times an antiheparin and more frequently, a tissue antithromboplastin (FIGURE 25).

In conclusion, it would appear that the syndrome of obstetrical fibrinogenoenia has many associated features that may differ from case to case, even hen the histological findings indicate a similar pathogenic mechanism. Also, nfusion of placental constituents, amniotic fluid (especially in sensitized aninals) and fibrinokinase, and the induction of hemorrhagic shock all lead to a emorrhagic syndrome with a fibrinogenopenic picture similar to that found in nost obstetrical accidents. The only exception is the development of a circu-

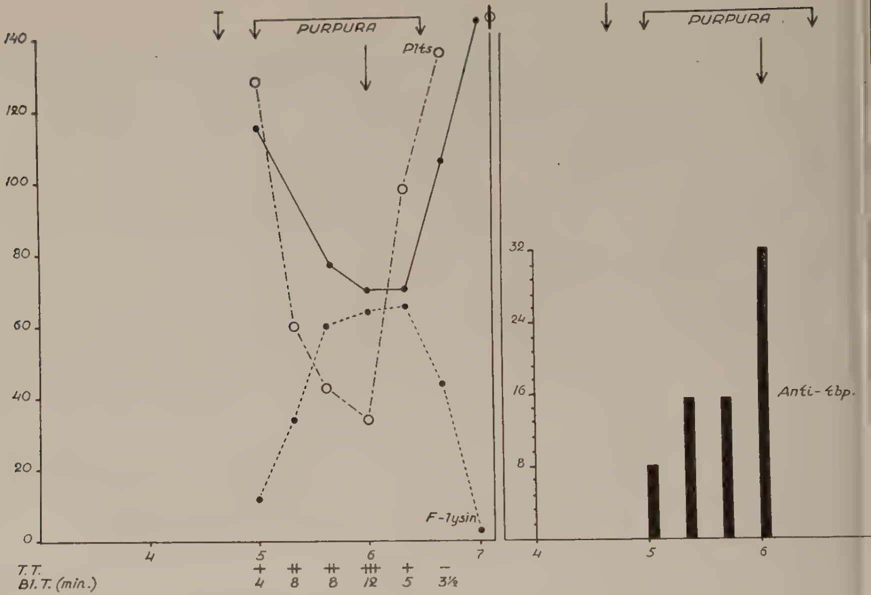

FIGURE 25. Intrauterine fetal death and hemorrhagic tendency. Evidence of feta death was first noted during the fifth month of pregnancy. Platelet count and plasm fibrinogen level fell, fibrinolysin increased, and a circulating anticoagulant (antithromboplastin) developed during the two following months, until the patient aborted spontaneously Purpura was frequent during the same period of time.

lating anticoagulant that appears typically after infusion of thromboplastin These various findings emphasize, in our opinion, the complexity of the etiological mechanism involved, and may suggest even that we are dealing with multiple syndromes. Perhaps the most exciting observation of our work i the fact that hemorrhagic shock may induce fibrinogenopenia, thrombocytopenia, and the histological findings of intravascular clotting. It remains to b established how much the fibrinogenopenia of many cases of separation o placenta may be secondary to the effects of shock originally induced by hemorrhage.

Summary

(1) An analysis of cases of fibrinogenopenia in pregnancy and delivery indicated the possibility of various pathogenetic mechanisms, including (*a*) intravascular clotting with secondary defibrination and thrombocytopenia; (*b* activation of the fibrinolytic system; and (*c*) the appearance of anticoagulant of various types. The possibility was raised that the fibrinogenopenic syndrome may encompass a number of different entities.

(2) An experimental study was carried out in an attempt to evaluate th relative importance of various mechanisms in the hemorrhagic accidents o pregnancy and delivery. As expected, the injection of amniotic fluid and uter

ine material into the circulation was followed by findings suggesting intravascular clotting. The administration of amniotic fluid caused more severe symptoms if the animal had been sensitized previously. Administration of placental thromboplastin caused massive intravascular clotting, accompanied by activation of fibrinolysin and by the appearance of hemolysis and of a circulating anticoagulant. The administration of tissue fibrinokinase induced primarily activation of fibrinolysin but also intravascular clotting, probably because of the inability to separate thromboplastic impurities. Hemorrhagic shock alone caused a bleeding abnormality whose picture was similar in most aspects to that produced by the infusion of placental thromboplastin.

It appeared that all factors considered could play a role in the etiology of the ibrinogenopenic accidents of pregnancy, their importance varying from case to case.

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COAGULATION COMPONENTS IN NORMAL AND ABNORMAL PREGNANCIES*

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The last few years have seen a remarkable change in the diagnosis and management of hemorrhage at childbirth. This change has resulted from an appreciation of the fact that excessive bleeding need not be due to localized abnormalities of the uterus or vagina, but rather to the presence of a generalized hemorrhagic state. It is our purpose to review briefly current knowledge concerning hemostatic mechanisms in normal pregnant and parturient women and the alterations that may occur in these mechanisms and result in severe and sometimes fatal bleeding.

During the course of a normal pregnancy a number of changes occur in the coagulative system of plasma. The concentration of fibrinogen—that is, of that protein from which the clot ultimately forms—gradually rises. The average concentration of fibrinogen in nonpregnant women is about 300 mg. per 100 ml. The concentration of fibrinogen rises to about 370 mg. at the thirteenth to sixteenth weeks, and to an average of about 440 mg. at the thirty-seventh week and thereafter.[1] The mechanisms responsible for the rise in fibrinogen are not clear. J. Rosenblum at Western Reserve University has attempted to link the rise to the known endocrinologic changes that occur during pregnancy, but thus far without clear-cut success. This rise in the concentration of fibrinogen is apparently responsible for the increase in the sedimentation rate of erythrocytes so characteristic of pregnancy.

At the same time there is a moderate increase in the concentration of prothrombin during pregnancy.[2] In a small series we[3] noted that the average concentration of prothrombin in pregnant women was 121 per cent that of nonpregnant women; more recently, Alexander *et al.*[4] have published similar figures. On the other hand, there is a striking increase in the concentration of the stable serum factors that accelerate the conversion of prothrombin to thrombin—that is, so-called proconvertin, pro-SPCA, or Factor VII.[4, 5] Moreover, we have been able to show that the concentration of Christmas factor, or plasma thromboplastin component, is increased in the plasma of pregnant women (FIGURE 1).[6] No better explanation for the increased concentration of prothrombin, proconvertin, and Christmas factor can be offered than for the increase in fibrinogen. All these factors require vitamin K for their synthesis, all are depressed in certain cases of hepatic disease, and the synthesis of all three is depressed by therapy with Dicumarol and similar agents. This suggests that a single mechanism will be found to be responsible for the increased rate of synthesis of the three factors, but the nature of the mechanism remains unknown.

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None of the other clotting factors for which one can perform reasonably quantitative assays is altered during normal pregnancy. The concentrations of antihemophilic factor,[6] proaccelerin,[4, 6] Hageman factor,[6] and platelets[3, 7] are all within normal limits relative to nonpregnant women. Morevoer, the various over-all tests of hemostatic function, such as for clotting time,[3, 8, 9] bleeding ime,[9] and tourniquet tests are all normal during pregnancy.

During normal labor and delivery, no significant alterations occur in the coagulability of peripheral blood.[3, 7] It is noteworthy that the clotting time is unaltered by parturition. This is of particular interest when one reads about alterations in clotting alleged to occur as the result of stress; it is hard to imagine a more emotion-packed experience than childbirth. There is, however, one change that occurs consistently. In the hours after delivery there is regularly an increase in the fibrinolytic activity of plasma.[3, 9] The time that elapses until clotted plasma dissolves—that is, the clot lysis time—regularly shortens, so that increased fibrinolytic activity is detectable several hours postpartum. Clot lysis time returns to normal within about 24 hours. This increase in ibrinolysis is minimal, and probably does not play any significant role in hemostasis. The cause of the rapid fibrinolysis is not known. The same increased rate of fibrinolysis can be seen in any normal individual after brief exercise or the injection of epinephrine, or accompanying any surgical procedure, but the process through which these events bring about increased ibrinolytic activity is unclear.

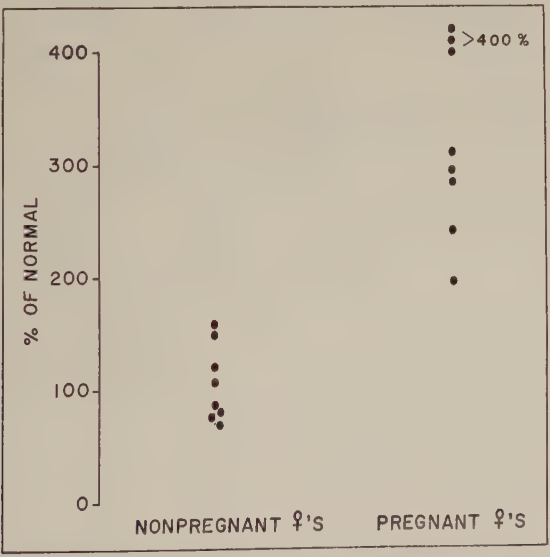

FIGURE 1. Christmas factor (PTC) activity of citrated plasma obtained from normal nonpregnant and pregnant women. The assay was performed by measuring the recalcified clotting time of a mixture containing 0.1 ml. plasma from a patient with severe Christmas disease, 0.1 ml. plasma to be tested (diluted serially with barbital-saline buffer), 0.1 ml. crude cephalin, and 0.1 ml. 0.025 M calcium chloride. The individual values are compared to the values obtained using a pool of plasmas of the nonpregnant women.

At least two factors are involved in the control of bleeding from the placenta site after delivery. Barnes[10] has emphasized the importance of uterine contraction. The contracting myometrium constricts the intrauterine blood vessels, and the flow of blood from the raw placental site is stanched. However, this means of stopping bleeding is not of itself adequate for permanent hemostasis. In addition, the patient must have an intact coagulative mechanism for, in patients whose blood is depleted in fibrinogen, uterine bleeding will soon appear, and may be of catastrophic proportions.

Under a number of different conditions, changes occur in the hemostatic mechanisms that may result in serious and even lethal uterine or generalized bleeding. Among these conditions are premature separation of the placenta, amniotic fluid embolism, the intrauterine retention of a dead fetus, criminal abortion, and eclampsia.

Premature separation of the placenta is probably the commonest cause o severe uterine hemorrhage associated with pregnancy. However, it is only occasionally accompanied by generalized bleeding, a phenomenon first observed by DeLee in 1901.[11] The nature of this generalized bleeding was elucidated by Dieckmann,[12] who demonstrated that the concentration of fibrinogen in the blood of such patients was drastically reduced. Presumably the uterine and extrauterine bleeding occurred because there was inadequate fibrinogen in the circulating blood to provide fibrin for the control of bleeding at sites of even trivial injury. In some cases the hypofibrinogenemia has been accompanied by decreases in the concentrations of prothrombin and proaccelerin in the plasma, by thrombocytopenia, and by excessive plasma fibrinolytic activity.[13]

There has been considerable speculation concerning the pathogenesis of the hypofibrinogenemia. One early view was that the fibrinogen was lost through hemorrhage and utilized in the formation of fibrin clots at the site of the placental separation. However, severe hemorrhage in other situations, such as intra-abdominal bleeding, is usually not associated with hypofibrinogenemia. Moloney *et al.*[14] and others have suggested that the fibrinogen is destroyed through the action of the proteolytic enzymes of the plasma itself. However, it has not been possible to demonstrate abnormal plasma fibrinolytic activity in patients with premature separation of the placenta and hypofibrinogenemia.[13, 15-17] This does not preclude the possibility that lysis of fibrinogen occurs elsewhere in the body than in the circulating blood. The hypothesis advocated by Schneider,[18] Page, *et al.*[15] and Weiner and his associates[19] seems most consonant with the available data. It has been known for almost a century that the intravenous injection of tissue particles—that is, of thromboplastic material will render the blood incoagulable. In 1921 Mills[20] showed that this incoagulability was accompanied by a decrease in the concentration of fibrinogen in the circulating blood. Presumably the injection of thromboplastin had resulted in intravascular clotting, with deposition of fibrin in the blood vessels throughout the body. In one such study the concentration of fibrinogen in the circulating blood fell rapidly[21] during the infusion of homologous thromboplastin. Several hours after the infusion was discontinued, fibrinogen reappeared in the circulating plasma, and its concentration soon returned to normal evels. Coincident with the hypofibrinogenemia, other alterations in the clot-ing mechanism were observed, namely, a prolongation of the clotting time, a lecrease in the concentration of prothrombin, a prolongation of thrombin ime—that is, the clotting time of a mixture of thrombin and oxalated plasma—hrombocytopenia and, under certain conditions, fibrinolysis.[21, 22] These events ear a close resemblance to the changes in the blood seen in premature sepa-ation of the placenta, and suggest that in this disorder the hemorrhagic state nay be the result of the entrance of placental tissue, known to be highly throm-oplastic, into the peripheral circulating blood. This viewpoint implies that bnormal fibrinolysis is either secondary to the intravascular coagulation or s a sequel to the shock that may accompany severe hemorrhage.

A second cause of a generalized bleeding tendency during childbirth is mniotic fluid embolism. In this condition, first described by Steiner and ushbaugh,[23] amniotic fluid enters the maternal circulation just before, during, r after childbirth. In some patients there is sudden respiratory distress and yanosis, followed within a few minutes by the death of the patient. Other atients seem to slip gradually into shock in the hours postpartum and, if ntreated, may die. Weiner and his associates[24] noted that in many of the eported cases there was severe uterine hemorrhage, sometimes associated with vidences of a systemic bleeding tendency. They found, moreover, that am-iotic fluid had thromboplastic properties. They therefore suggested that in hese patients the amniotic fluid that entered the maternal circulation caused ntravascular clotting and hypofibrinogenemia, just as in the case of premature eparation of the placenta. Uncontrollable hemorrhage and death would thus esult because of the failure of the normal hemostatic mechanisms. Vosburgh nd one of us[25] had the opportunity to study a patient with this condition and ound, as Weiner had predicted, that the bleeding tendency was associated with evere hypofibrinogenemia. The patient also had a long thrombin time, a lecrease in the concentration of prothrombin, mild thrombocytopenia, and xcessive plasma fibrinolytic activity. The same changes have now been ob-erved in other patients with this disorder.

At first nearly all patients with this disorder died. Our more recent experi-nce has been more favorable. We believe several factors are responsible. irst, the cases are recognized at an earlier time, so that replacement therapy ith fibrinogen can be instituted before blood loss is excessive. Second, opera-ive intervention in search of a site of bleeding has been discouraged and, finally, e use as little blood bank blood as possible, but give the patients freshly drawn lood as soon as it is feasible to do so.

A third disorder of pregnancy is the hemorrhagic state associated with the ntrauterine retention of a dead fetus. It has been recognized for many years hat hemorrhage is one of the major complications of missed abortion when abor ultimately takes place. In 1950, Weiner *et al.*[26] suggested that uterine emorrhage under these conditions might result from an alteration in the oagulability of the blood. Among 15 patients with intrauterine retention of dead fetus, they noted 3 in whom there was more or less severe uterine leeding. In each of the 3 there were also evidences of a systemic hemorrhagic disorder, for each had large apparently spontaneous ecchymoses, and 1 patient bled persistently at the sites of hypodermic injection and venipuncture. Weiner and his associates showed that the patient's peripheral blood was deficient in fibrinogen, and they postulated that the bleeding tendency was the result of this hypofibrinogenemia.

Since Weiner's original report, many other cases have been described. On the basis of these cases and our own unpublished experience, certain generalizations can be made. First, hypofibrinogenemia in patients with missed abortion is not a rarity. For example, in one study this abnormality was observed in 5 of 28 patients with retained dead fetus who were studied prior to the appearance of a bleeding tendency.[27] Second, the bleeding tendency has been observed in women in whom fetal death has taken place as early as the sixteenth week and as late as the thirty-first week after the onset of the last menstrual period. Third, the cause of fetal death has not been shown to bear a relationship to whether or not hypofibrinogenemia will ensue. Fourth, hemorrhage may occur as early as three weeks after the cessation of fetal movements, that is, considerably earlier than has been reported previously. Fifth, a decrease in the concentration of fibrinogen may be only one of several coagulative defects present; moderate decreases in the concentration of prothrombin and the platelet count also have been observed. Finally, there is evidence that the fall in the concentration of fibrinogen may be gradual, so that one can predict the appearance of a bleeding tendency In the first three cases reported by Weiner, the presence of a hemorrhagic disorder was discovered only at, or just before, delivery. However, in some patients tested at weekly intervals after the apparent death of the fetus, the concentration of fibrinogen gradually fell from normal values to abnormally low levels until a point was reached at which systemic bleeding would occur. The critical level seemed to be about 100 mg. per 100 ml. of plasma. In each patient, emptying the uterus resulted in prompt correction of the defect. In our patients the decline in the concentration of fibrinogen has been gradual. This need not always be the case; Hodgkinson and his associates[28] have described instances in which the concentration of fibrinogen fluctuated between normal and depressed values in the weeks prior to delivery.

The mechanisms responsible for the development of this remarkable condition are entirely obscure. Four possibilities have been considered. First, it has been suggested that there is impairment of the synthesis of fibrinogen. This hypothesis implies that the contents of the uterus in some way depress the synthesis of this protein, for the concentration of circulating fibrinogen rises within a matter of several hours after the uterus is emptied. This is an attractive hypothesis, since there is evidence that the concentration of fibrinogen is under hormonal control. For example, pituitary growth hormone is said to increase, and cortisone to depress the concentration of fibrinogen. It is conceivable that hypofibrinogenemia associated with missed abortion is due to an endocrinologic influence upon the synthesis of this protein. However, no evidence has been adduced that synthesis is actually depressed.

Second, it has been suggested that the fibrinogen in the plasma has in some way been destroyed. For example, Vecchietti[29] believed that the amniotic fluid surrounding the dead fetus was fibrinolytic. However, it has not been possible to confirm this observation. Nor is there evidence that the fibrinogen is destroyed by the fibrinolytic activity of the plasma. Third, there is the possibility that the fibrinogen has been so altered that it is no longer able to form a fibrin clot. Against this view is the fact that the deficiency in fibrinogen can be demonstrated electrophoretically and chemically. Finally, Weiner and his associates[26] suggested that the hypofibrinogenemia was due to intravascular defibrination resulting from the entrance of thromboplastic material into the maternal circulation. This thromboplastic material presumably originates from the products of conception. A serious stumbling block to this theory is that it is hard to explain how the thromboplastic material would be introduced into the maternal blood stream steadily enough to produce that orderly decline in the concentration of fibrinogen that sometimes occurs. Thus no acceptable explanation has been offered for the bleeding diathesis associated with intrauterine retention of a dead fetus.

A fourth syndrome is one that to our knowledge is totally unexplained, namely, severe hypofibrinogenemia or afibrinogenemia in cases of criminal abortion. We are familiar with reports of four cases in the literature.[13] In all four cases the patients died despite intensive therapy. The hypofibrinogenemia was accompanied by thrombocytopenia in three instances, by prolongation of the thrombin time in one case, and by excessive plasma fibrinolytic activity in another. A most intriguing part of this syndrome is the fact that three of the patients had bacteremia, in two cases with coliform organisms, and in the third with *Clostridium perfringens*, *Streptococcus fecalis*, and *bacteroides*. The relationship of the bacteremia to the hypofibrinogenemia is not clear. In a study performed in association with Conley *et al.*[30] we were unable to produce hypofibrinogenemia in rabbits injected with the organism isolated from one of these patients.

Finally, we wish to mention briefly the abnormal bleeding in patients with pre-eclampsia and eclampsia. These disorders are not ordinarily thought of as syndromes associated with clotting defects yet, as Kennan *et al.*[31] recently re-emphasized, bleeding, particularly into the central nervous system, is a frequent cause of death, and hemorrhagic phenomena are commonplace. In a recent study with Weisman *et al.*[32] we were surprised to find that hemostatic defects were present in 29 of 31 patients with eclampsia or severe pre-eclampsia. In 19 of 27 patients the clotting time of whole blood, measured in silicone-coated tubes, was prolonged. The thrombin time—that is, the clotting time of a mixture of the patient's oxalated plasma and bovine thrombin—was abnormally long in 27 of 31 cases. Thrombocytopenia, sometimes of great severity, was found in 8 of 28 patients, including 7 with eclampsia. In a few of the patients generalized bleeding manifestations such as epistaxis, cutaneous ecchymoses, petechiae, and hematuria were present. The pathogenesis of these various changes is unknown. It is noteworthy, however, that many of these patients had evidences of a hemolytic anemia, and thrombocytopenia is frequent in nonobstetric as well as obstetric cases. Unfortunately, this correlation does not help us decide which mechanisms are involved.

Although these five clinical syndromes—premature separation of the placenta, amniotic fluid embolism, intrauterine retention of a dead fetus, crimina abortion, and toxemia of pregnancy—are fairly well defined clinically, ou knowledge concerning the pathogenesis of their hemorrhagic complication seems meager. It is to be hoped that the next few years will bring som clarity to this problem so that our therapeutic efforts can be more rationa and perhaps more rewarding.

Acknowledgments

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ETIOLOGY OF FIBRINOPENIA: FIBRINATION DEFIBRINATION

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Fibrination is coagulation within, and of, the *circulating* blood. Naturally defibrination (that is, fibrinopenia) develops during the fibrination.

Among reported instances of fibrinopenia, more than one half develop during acute profound disorders peculiar to late pregnancy.[3] These obstetric disorders, in turn, are complications secondary to the peculiarities and requirements of human placentation. The most extensively studied example, both a to incidence and as to its mechanism of production, is found in abrupti placentae.

In abruptio, autoextraction of tissue-thromboplastin into the maternal circulation (FIGURE 1), can cause fibrination (FIGURE 2). Fibrinogen disappear from the blood (TABLE 1). Extensive fibrin deposits of a characteristic kind to be carefully distinguished from thrombi and from emboli (FIGURE 3), can be demonstrated microscopically (FIGURES 4, 5, 6, and 7). Fibrination-defibrination not only can, but does, occur.

Causing trauma experimentally to the placental attachment in the pregnant rabbit results in similar findings; this is in some way comparable to the premature separation of human abruptio placentae.[72] Not only may the anima go into shock; if it survives it may develop "incubated lesions" comparable t those of toxemia of pregnancy; if it dies at once, the fibrin occlusions, characteristic of fibrination, can be found occluding the pulmonary vascular bed (FIGURE 8). These fibrin deposits lead to the interpretation that tissue-thromboplastin was released during the experimental premature placental separation. In addition, embedded within the fibrin occlusions are trophoblast cells that provide visible evidence that uteroplacental materials were introduced into the circulating blood at the same time that the fibrination occurred (FIGURE 9).

The fibrin deposits of fibrination can be studied more directly when laboratory extracts of tissue-thromboplastin are injected into experimental animals. Fibrin occlusions from thromboplastin injection form at once and are characteristic (FIGURES 9 and 10). When extensive, these can and do cause syncope, convulsions, and death within minutes. Among survivors, incubated lesions comparable to those of toxemia of pregnancy can develop.[14, 66, 67, 72, 73] These extraordinary fibrin depositions can cause acute cor pulmonale and shock of acute cor pulmonale, as demonstrated later by diverse monitorings, including kinetograph recordings both of the hyperacute development of the acute cor pulmonale and of substantial recovery, within a matter of minutes, during the interval of observation.

Last, but not least, the fibrin occlusions can disappear (FIGURE 11); perhaps they are lysed away by some kind of fibrinolytic activity.

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[*] The work described in this report was aided in part by Grant H-3194 from the National Heart Institute, Public Health Service, Bethesda, Md.

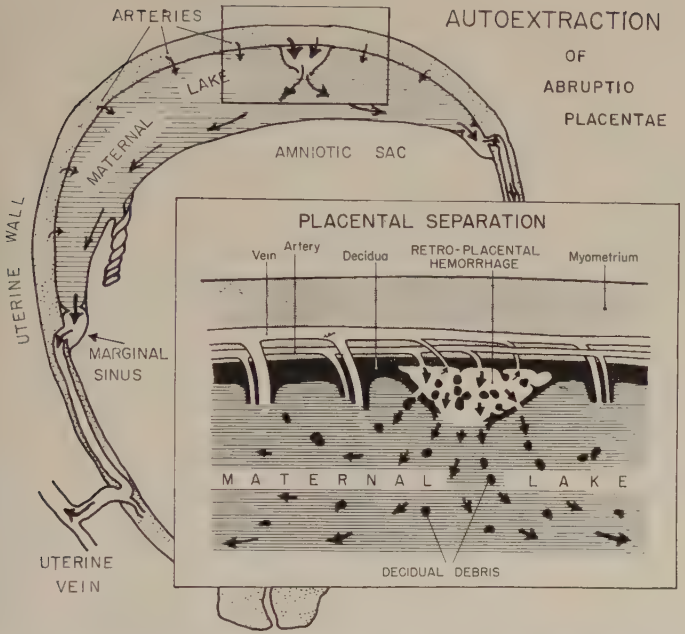

FIGURE 1. Probable mechanism of autoextraction of decidual tissue into the maternal circulation during abruptio placentae: an intradecidual hematoma forms within the retroplacental regions supplied by arterial flow, and expands under a force comparable to that of a hydraulic pump. The peculiarity of the expanding hematoma is that, when it ruptures, its contents flow into the general circulation via the maternal intervillous lake, carrying with it a decidual extract. From Schneider.[83]

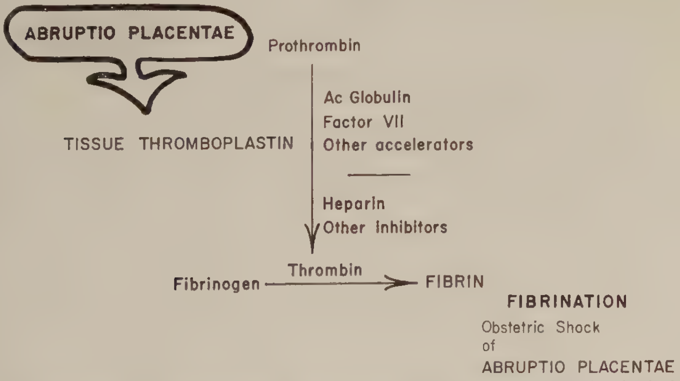

FIGURE 2. Initiation of coagulation in fibrination. Tissue thromboplastin is introduced nto the circulating blood by some accident, such as that of abruptio placentae, and initiates oagulation as in the two-stage reaction of Morowitz (as here shown, modified after Seegers).

Tissue thromboplastin, like other foreign materials, is soon removed from the circulating lood, because tissue thromboplastin can cause coagulation within, and of, the circulating lood, that is, it can initiate fibrination.

Table 1
Fibrinogen, Mg. Per Cent*

Patient	Lowest observed during abruptio	Calculated including transfusions	Final observed	Restored by patient
V. S. (W. H.)	60	200	420	220
P. M. (H. K. H.)	90	220	520	300
From Dieckmann[13]	60	140	440	300

Defibrination (defibrinogenation) and regeneration of fibrinogen in abruptio placent within one day.

* Modified after Schneider.[73]

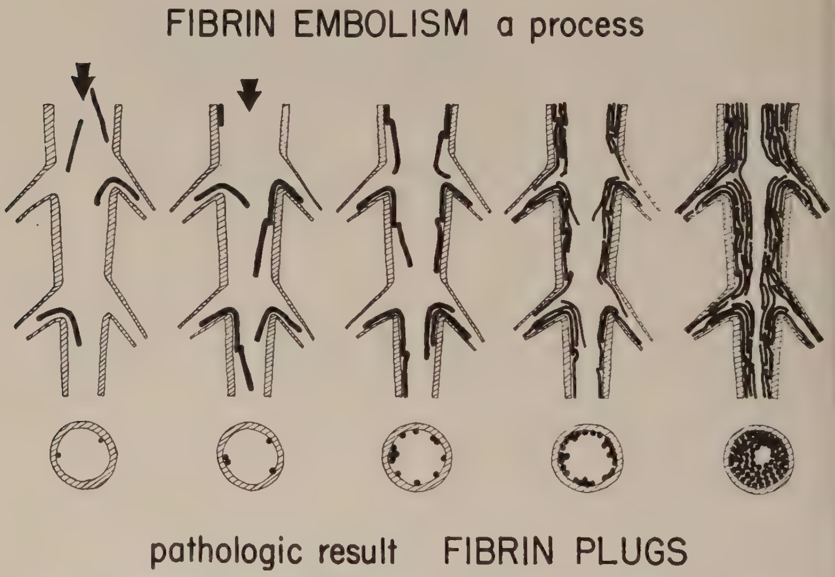

Figure 3. Animated diagram of fibrination. To initiate fibrination, it is necessary t initiate coagulation of fibrinogen within and of the circulating blood. Filaments of fibrin become attached to the vessel wall, possibly by becoming straddled over bifurcations of the vessels. Additional filaments of fibrin deposit out onto the pioneer fibers. The fibrin fibrilla are oriented in the lines of flow by the force of the blood stream. As more filaments of fibrin are added, a cast of the vascular tree is built up of fibrin and may progress to occlusion of the vessels. When the cast occludes at one level, a central core of whole blood may become en- trapped and form a whole-blood clot at other levels. The process is hyperacute and is to be distinguished from thrombus formation. Tissue thromboplastin, but not platelet thrombo- kinase, is qualitatively capable of initiating fibrination. Reproduced from Schneider.[83]

Some Possible Mechanisms of Production of Fibrinopenia

Variable degrees of fibrinopenia or of "defibrination" may develop if a insufficient amount of fibrinogen is produced to keep up with the bodily "turn- over" requirement for fibrinogen.

Infection severe enough to cause an inadequate production of plasma pro- teins, whether of (a) viral origin, for example, acute yellow atrophy of the liver, or (b) bacterial origin, for example, puerpural septicemia, may permit depletion of the enzymes of coagulation, as well as depletion of the substrate fibrinogen (for example, prothrombin determination may serve as a measure of liver dysfunction). Fortunately, maternal infections are usually controlled readily and hence do not cause significant disorders of coagulation. Congenital afibrinogenemia is of rare occurrence; because the deficiency is limited to fibrinogen alone, the individual is relatively free from hemorrhagic tendencies.[2] Hemostasis can be maintained by the remaining coagulation and thrombus-forming mechanisms. Menses may be normal.[41] It is not known whether, with congenital afibrinogenemia, uterine implantation of the ovum can occur, or whether pregnancy can develop and continue to term.[41] Conceivably, idiopathic fibrinopenia may occur.

Acutely acquired fibrinopenia can be caused by excessive removal of fibrinogen in some way. For example, fibrinogen can be used up rapidly,[20, 49, 65, 73, 74] as in fibrination-defibrination. Fibrination may develop during abruptio placentae of late pregnancy, during labor to deliver a hydatid mole, during missed abortion of late pregnancy, or it can be produced experimentally. Although the data in relation to pregnancy are far from convincing, conceivably fibrinogen may be destroyed rapidly, for example, by fibrinolysis, as perhaps under the influence of extreme activation of fibrinolysin. Curiously, it is probable that, during experimental fibrinolysis, as during fibrination, the enzymes of coagulation (as well as the substrate fibrinogen) tend to be severely depleted, thus raising the question of whether here too, the process proceeds stepwise: fibrination-defibrination.

The possibility cannot be excluded that appreciable fibrinopenia can be brought about by the introduction of foreign particles into the circulation. Indeed, certain plasma expanders may present this hazard. Whether there is coagulation to fibrin or simple adsorption of fibrinogen[37] onto the particles is a question open to investigation. Thrombocytopenia follows introduction of particulate debris into the circulation as, indeed, thrombocytopenia follows the introduction into it of diverse materials.

The possibility is sometimes considered that fibrinogen could be removed in whole blood clots or by its incorporation into true thrombi within special foci as, for example, in a retroplacental hematoma,[14] or in intervillous, placental thrombi,[101] a concept that is hardly convincing.[73, 74, 81]

Fibrinolysis

Profibrinolysin, a circulating precursor in the blood, can be activated to ibrinolysin by various agents, among them chloroform; the concentration required is not compatible with life. Among the notably more physiological activators are tissue fibrinokinase, urokinase, and streptokinase. A mechanism for the removal of fibrinokinase is present in the body. Likewise, there occurs an inhibitor of fibrinolysin in the plasma[57, 91, 97] and in the platelets.[32]

Fibrinolysin is still far from being a proved cause of fibrinopenia for abruptio placentae as originally suggested by Moloney *et al.*,[48] or of this or other disorders of late pregnancy, as suggested by other interested investigators.[106]

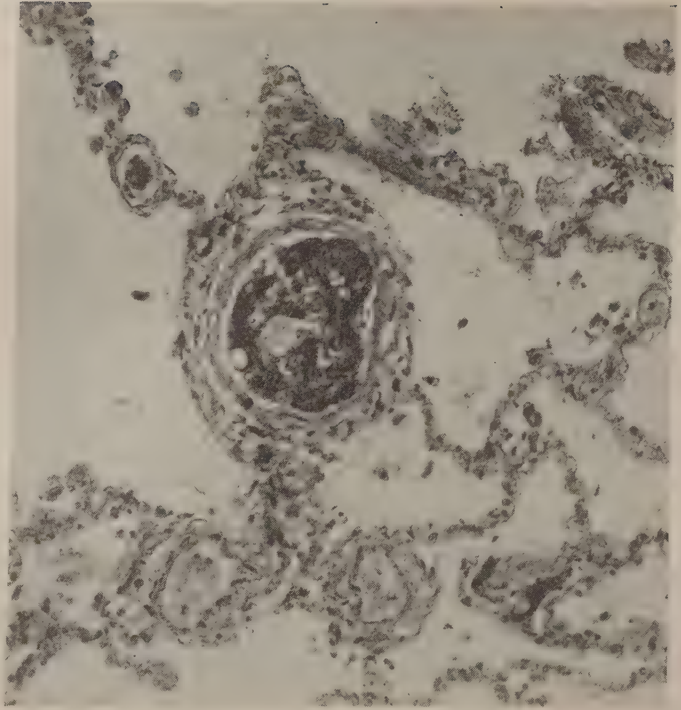

FIG. 4

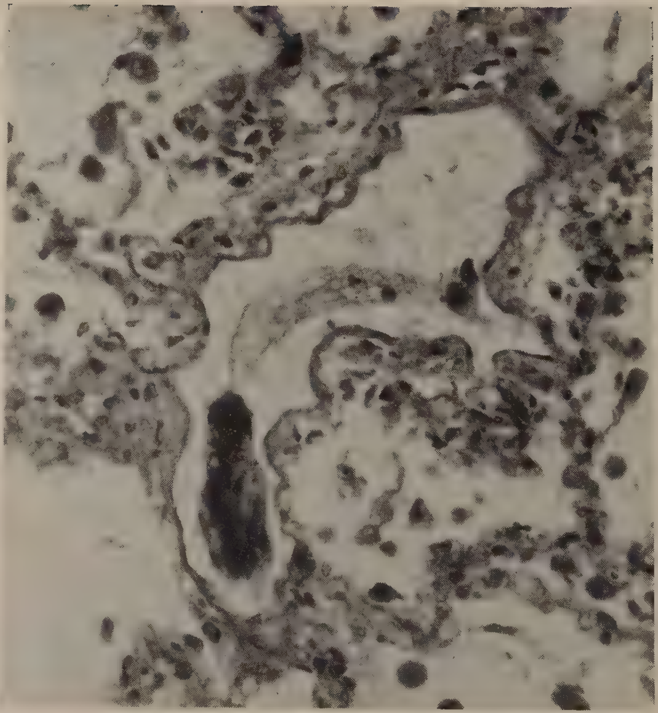

FIG. 5

FIGURES 4 to 7. Stained microscopic tissue sections provided by Bartholomew *et al.*,[3] all from the same patient, in whose tissues innumerable such fibrin deposits are present.

FIGURE 4. Low-power microscopic view of the lung of a woman recently dead of abruptio placentae. Four fibrin occlusions are visible in branches of the pulmonary arterial tree, the largest (center) showing a cross section of a fibrin occlusion in a small artery. The apparently solid mass is made up of innumerable fibrin threads here seen from end view; only in the central core is there a clot of whole blood. Compare this structure of the fibrin cast and its central blood clot with the schematic diagram of FIGURE 3. Reproduced from Schneider.[74]

FIGURE 5. Longitudinal view of a small fibrin deposit in a minute pulmonary artery under high-power magnification. A portion of the fibrin occlusion is cut in a sufficiently thin tangential section to illustrate the orientation of the filaments of fibrin in the

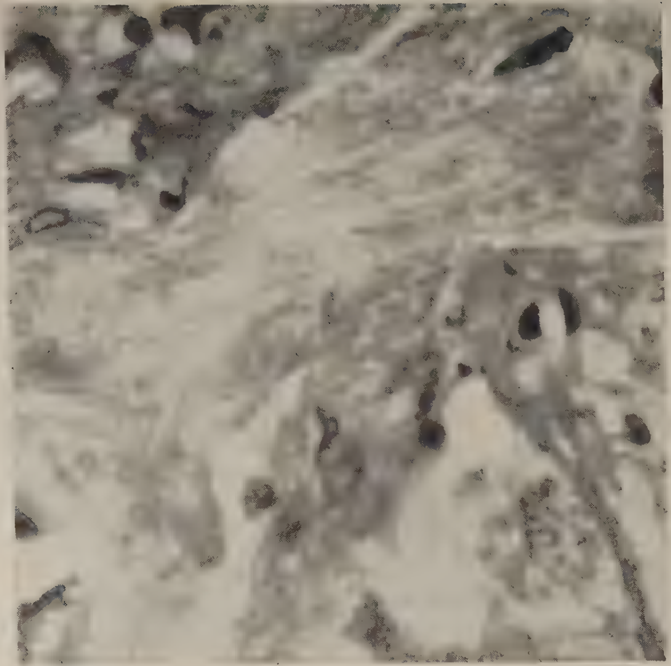

Fig. 6

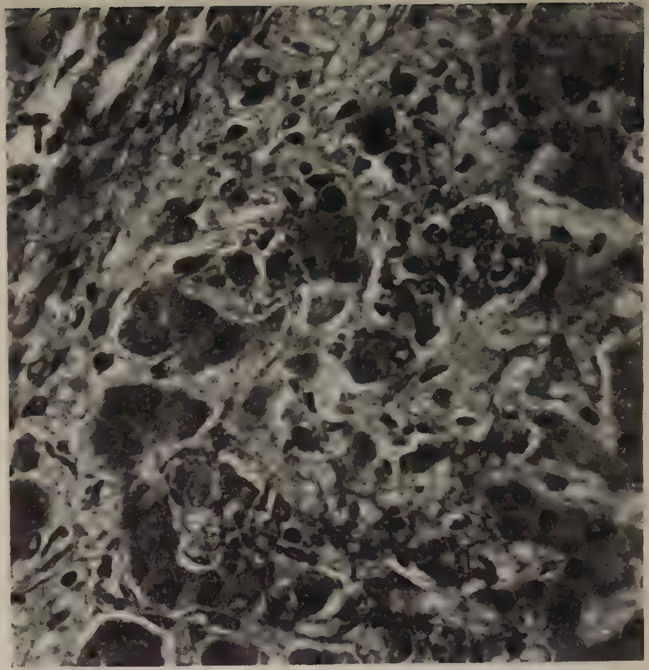

Fig. 7

Figure 6. Oil-immersion microscopic magnification of a characteristic fibrin occlusion that completely fills an afferent arteriole of a renal glomerulus; the capsule of the glomerulus extends downward in the right lower corner.

Figure 7. High-power magnification of region of periportal hepatic necrosis to show sinusoidal fibrin deposits.

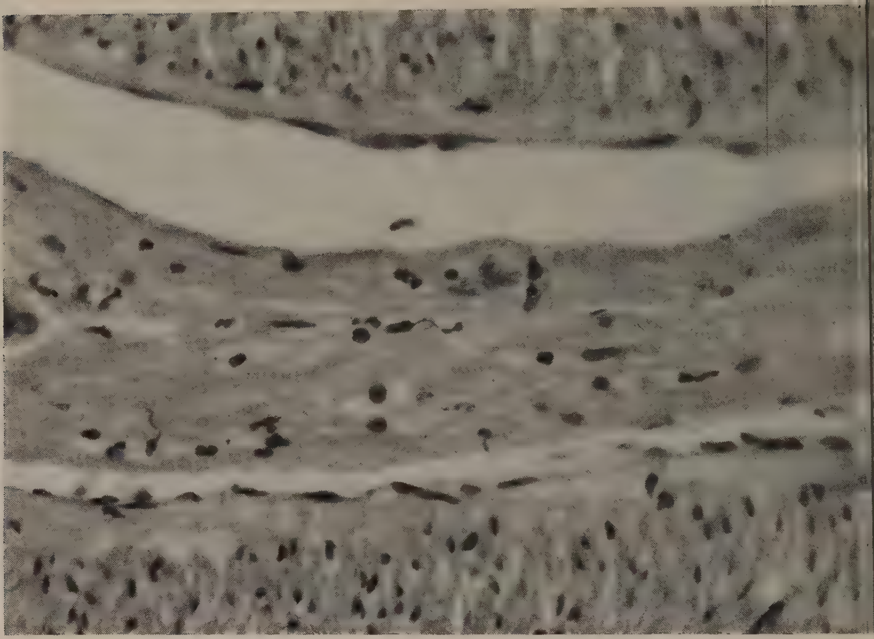

FIGURE 8. High-power microscopic view of a tissue section of a small pulmonary artery o a rabbit that died hyperacutely after experimental traumatic placental separations. Withi the artery there is a fibrin occlusion characteristic of fibrination (not a blood clot, not a throm bus). The fibrin filaments are deposited parallel to the lines of flow. The nuclei of some o the entrapped leukocytes are distended many diameters in the lines of flow. This demon stration of this fibrin occlusion, immediately following placental trauma, is consistent wit the interpretation that tissue thromboplastin was released into the maternal circulation an initiated fibrination. Three multinucleated trophoblast cells, here demonstrated microscopically within the fibrin deposit, one in the center of the photograph, the other two a either end, provide visible evidence that materials did indeed enter the maternal circulatio at the time of the placental trauma. Tissues fixed in alcohol to lyse erythrocytes. Thi tissue section is from the same pulmonary vascular occlusion as in FIGURE 7 in Schneider,[7] but is shown at a different level.

However, during surgery,[9] especially for certain neoplasms,[59] excessive fibrinolytic activity may develop and conceivably, by causing fibrinogenolysis, this could play a significant role in causing fibrinopenia. Whether, in the circulating blood, fibrinolysin can be activated by the release of tissue fibrinokinase, is a problem still to be resolved. Because of the occurrence of much fibrinokinase in the gravid uterus, it has been suggested[55] that fibrinokinase materials may be released in an effective form from the damaged conceptus into the maternal circulation, there to activate fibrinolysin and initiate fibrinolysis or, more pertinent to present considerations, to initiate fibrinogenolysis.

Contrariwise, circumstantial evidence implies that there is activation of some kind of fibrinolysin by a physiological protective mechanism following fibrination.[74, 81] Early removal of fibrin deposits that follow injection of tissue-thromboplastin was probably first noted in experimental animals by Thomas.[10

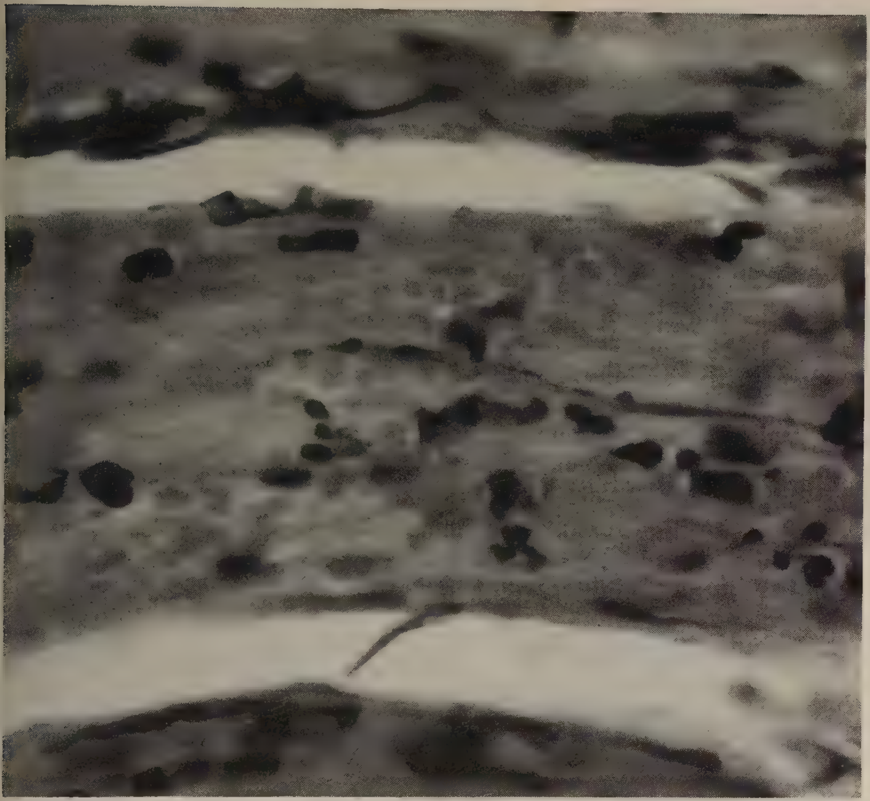

FIGURE 9. High-power view of longitudinal section of a fibrin occlusion in a small pulmonary artery of a rabbit that died hyperacutely following injection of placental-tissue-thromboplastin extract. The fibrin is oriented in the lines of flow. The nuclei of most of the entrapped leukocytes are distended to variable degrees in the lines of flow. One polymorphonuclear leukocyte is stretched across most of the microscopic field, with its lobes like beads on a string. This enlargement is from the same tissue section as in Schneider.[73]

This helps, at least in part, to explain an old mystery of why fibrin was not found after intravascular injection of an initiator of coagulation into experimental animals. The spontaneous disappearance of the characteristic fibrin deposits of fibrination would seem to require at least, potent focal fibrinolytic activity. It is noteworthy that disappearance of the fibrin deposits provides evidence of a fibrinolytic, but not necessarily of a fibrinogenolytic, activity.

Whether significant activation of circulating fibrinolysin may actually occur in late pregnancy, in the sense of causing fibrinogenolysis as distinguished from fibrinolysis, appears on careful analysis to be open to considerable doubt. Jackson *et al.*[30] demonstrate that disappearance of a clot formed from drawn venous blood in a test tube need not be interpreted as proof of fibrinolysis. Their investigations include patients with severe fibrinopenia of abruptio placentae. "Fragmentation" of a clot correlates with the degree of fibrino-

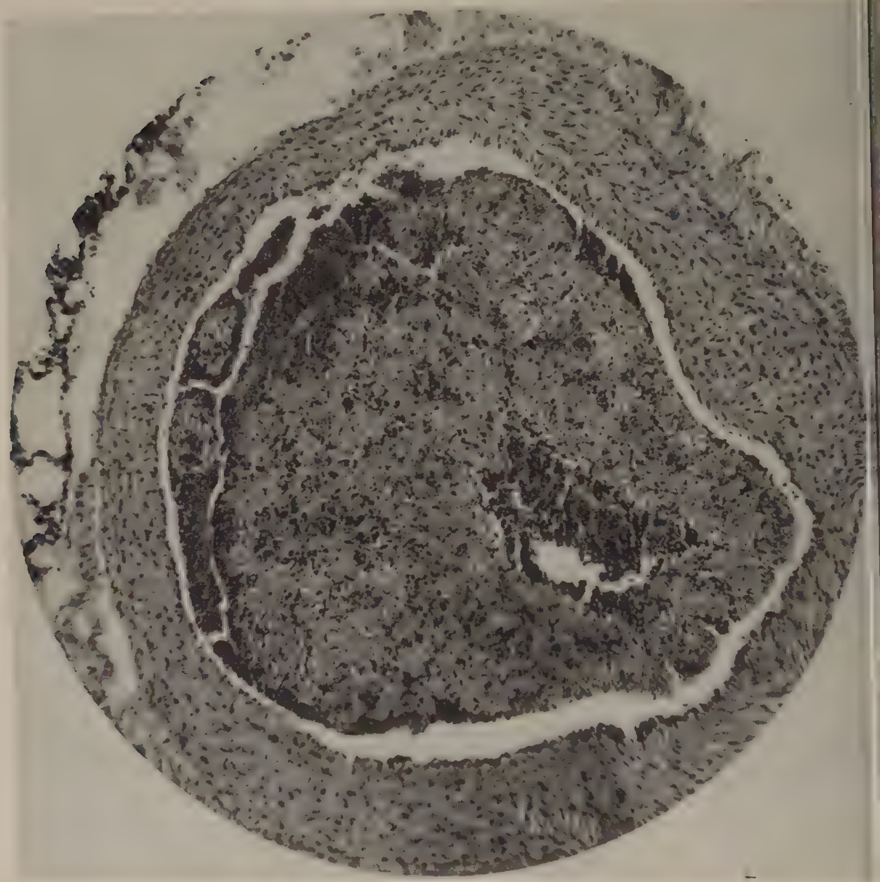

Figure 10. Cross-sectional view of a fibrin occlusion that forms a cast within a small pulmonary artery of a rabbit after injection of placental-tissue thromboplastin. The fibrillae of fibrin are oriented in the lines of flow and hence are seen here as myriads of separate points; compare with cross section in schematic diagram of FIGURE 3. A whole-blood clot is entrapped somewhat eccentrically within the cast of fibrin.

penia, and hence correlates better with the interpretation that the clot had only a low fibrin content to begin with; that is, that it was formed from already fibrinopenic blood. Ratnoff *et al.*,[61] through carefully devised analyses, were unable to demonstrate fibrinolytic activity in drawn venous blood from a patient with the dead fetus syndrome; Ratnoff and Vosburgh[62] did find fibrinolytic activity in plasma at high dilutions from the drawn, venous blood of a patient in shock from amniotic fluid embolism (there was also excessive "thrombin-inhibitory" activity). Lewis *et al.*[44] observed lysis of a plasma clot of an abruptio patient with only 35 mg. per cent of fibrinogen.

Phillips *et al.*,[56] using special methods, demonstrated small but definite fibrinogenolytic, and greater fibrinolytic, activity in the blood of several obstetric patients, especially when there were unusually low fibrinogen levels

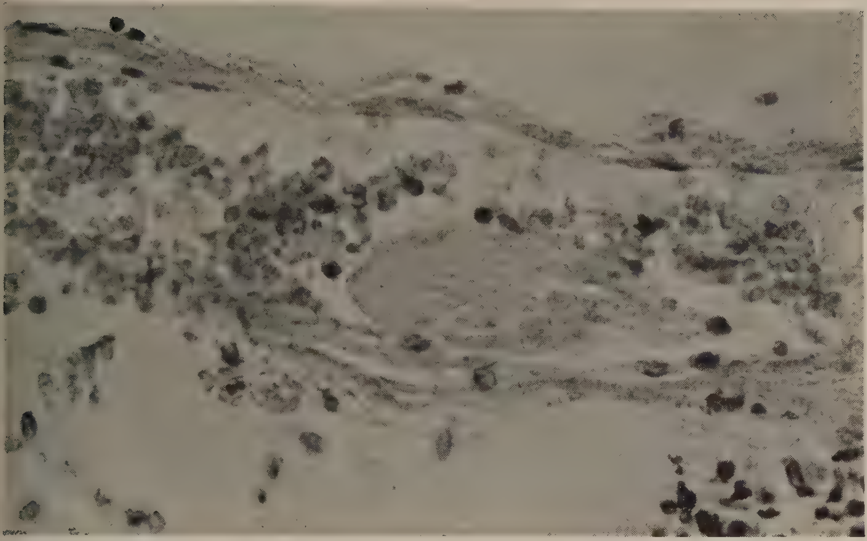

FIGURE 11. Amorphous deposit in a small pulmonary artery in the lung of a woman who died five days after abruptio placentae. Whether this is a remnant of a fibrin occlusion now largely lysed away cannot be determined. From Schneider.[74]

They mention that after deposition of fibrin (fibrination) there must be activation of a fibrinolytic enzyme system.

Jackson *et al.* note that if the fibrin meshwork of a clot is scanty, erythrocytes may more readily fall out of the clot. The remaining button of fibrin may retract markedly, the more markedly the less dense the clot, just as characteristically, a less dense plasma clot may retract more markedly.[57, 78] The minute, contracted button of fibrin from the blood clot may readily become lost among the sedimented erythrocytes, there to remain unobserved, and perhaps not lysed. With visible fixation of the button to the wall of the test tube or with flotation of the button of fibrin by entrapped bubbles Jackson *et al.*[30] were able to observe such a coagulum continuously in their studies; it did not lyse. In a parallel study for lysis of plasma clots, in contrast to lysis of blood clots, of abruptio, of the dead fetus syndrome, and of amniotic embolism, these investigators did not observe lysis.

Although, in purified systems, fibrinolysin may attack both fibrin and fibrinogen, the antifibrinolysin mechanism in the blood protects dissolved fibrinogen itself from attack by fibrinolysin. This does not prevent attack upon coagulated fibrin; indeed, fibrinolysin tends to become absorbed onto fibrin, and there it may actively attack the fibrin. Consistently, in experimental rabbits, fibrinolysin, activated in the circulating blood, was not found significantly to destroy fibrinogen.[31] Thus, in the body the fibrinolysin-antifibrinolysin system appears to be well adapted to removal of fibrin formed during fibrination, but has a physiological mechanism acting to prevent or to minimize fibrinogenolysis.[83]

In contrast, Jorpes considers that overwhelming activation of fibrinolysin may occur, for example, by potent bacterial streptokinase activation during human pulmonary surgery; Albrectsen,[1] in Astrup's laboratory, and others have been able to produce such overwhelming activation of fibrinolysin as to cause fibrinogenolysis.

In summary, whether significant fibrinogenolysis, as distinct from the fibrinolysis that follows fibrination, occurs in relation to obstetric complications remains to be demonstrated with satisfactory assay methods such as those used by the several investigators quoted above. In the blood, fibrinolysin ordinarily attacks fibrin, not fibrinogen. In diverse conditions, *in mortuo*[10] as well as *in vivo*, the steps of defibrination are: first, coagulation of fibrinogen to fibrin; second, lysis of the fibrin.[83] As in experimental fibrination in animals, so also in the fibrination of observed obstetric disorders, fibrinolysin seems to play an active role in the physiological removal of fibrin that has been deposited by fibrination.[83]

"Fibrination" of Extreme Shock or of Collapse Versus Fibrinolysin

A small residuum of maternal deaths or of severe complications with special or obscure disturbances of coagulation[30, 39, 44, 56, 65] grows more conspicuous as deaths from other causes become rarer. Reports gradually accumulate confirming the view that defibrination may occur, and this is not always related to or at the time of abruptio placentae; sometimes it follows simple, massive hemorrhagic shock. The exsanguinating hemorrhage may be secondary to abruptio or to placenta previa; it may be associated with cervical laceration; or it may be due to simple age-old obstetrical problems such as an inadequately or slowly contracting postpartum lower uterine segment. According to both published and privately reported cases, after extensive hemorrhage there may then supervene, not merely the fibrinopenia or partial defibrination so characteristic of autoextraction of abruptio placentae, which is discussed above, but essentially complete defibrination.

As has been discussed elsewhere,[83] in collapse or agonally spontaneous fibrination-defibrination can become essentially complete (perhaps this develops because of the extreme shock, or because of anoxia accompanying traumatic shock, yet under conditions wherein the blood still circulates to at least some degree through the tissues for a time). This low-grade fibrination-defibrination, like the massive process initiated by tissue-thromboplastin,[74] appears to proceed first by coagulation, and then by lysis of fibrin.[83] Here spontaneous activation of fibrinolysin apparently occurs through some inherent bodily mechanism, just as it does in some way following injection of tissue-thromboplastin. Perhaps an extreme dramatization is provided at a pragmatic level in the cadaver blood bank of Yudin.[109] In the bodies he selected, shortly after death the blood had already coagulated and the fibrin within it had already lysed away, in that order. There was no need to add anticoagulant to the blood while it was being collected. Presumably fibrinolysin had been activated agonally; post-mortem fibrin formed gradually and was lysed away as it was formed. There is no reason to assume that the fibrinolysin had attacked the fibrinogen itself; when Yudin collected the cadaver blood too soon, it coagulated in the container, but this was no great inconvenience, because the fibrin soon lysed spontaneously. Here too, then, the fibrinolysin attacks fibrin, not fibrinogen.

Tissue-thromboplastin is not available to activate the "agonal" fibrination-defibrination that occurs in collapse; however, since the blood comes to rest, platelet thrombokinase could initiate a form of low-grade fibrination.

The lack of fibrinogenolysis in this low-grade agonal fibrination-defibrination leads, as it does after injection of tissue-thromboplastin, to the suggestion that the activation of fibrinolysin may be more a physiological than a pathological process, for here, too, the activated fibrinolysin serves to lyse intravascular occlusions.

To summarize: it is plausible that in severe shock or in collapse a low-grade form of fibrination-defibrination that can nevertheless approach afibrinogenemia may be initiated during relative stasis of the blood. Either because of this low-grade fibrination or because of the shock itself, fibrinolysin seems to become activated in some way, even as it does following fibrination from tissue-thromboplastin. In general, fibrinolysin activated under these conditions attacks fibrin in precisely the same way that it attacks fibrin when activated under other conditions; essentially it seems not to attack fibrinogen.

We must not overlook the hazard that appreciable activity of a lysin that attacks fibrin, even if it be prevented from attacking fibrinogen by a circulating antilysin, may conceivably still contribute to a hemorrhagic diathesis: the fibrin, once formed, may become susceptible to the fibrinolysin. Hence, thrombi for hemostasis, especially thrombi formed in the presence of the lysin, may conceivably be susceptible to lysis. To what degree this hazard may be ameliorated by the presence of the antifibrinolysin present in platelets themselves, which contribute to the thrombus, is not known and needs to be investigated.

Fibrination Defibrination

During "fibrination,"[17] that is, during coagulation of fibrinogen within and of the circulating blood, fibrin is formed in, and is deposited out of, the blood. Fibrination can be mediated by tissue thromboplastin (FIGURE 12). Coagulation, but not fibrination, can be caused by platelet thrombokinase under presently known conditions (FIGURE 12). For platelet thrombokinase to cause coagulation, as in a platelet thrombus, it requires freedom from phagocytosis; in the circulating blood, platelet fragments do not seem to survive long enough to initiate significant coagulation. Platelet-thrombokinase can cause coagulation in blood at rest, or in blood removed from the circulation, according to a process long investigated in much detail and shown in abbreviated form in FIGURE 12. This is a significant mechanism for hemostasis. Consistent with the requirement for survival that the circulating blood remain fluid, even though coagulable, the wisdom of the body is such as to prevent the platelet-thrombokinase mechanism from causing significant coagulation in, and of, the circulating blood; that is, it suffices to prevent fibrination.[83, 89]

With regard to the latter, except in the most extreme degrees of fibrination

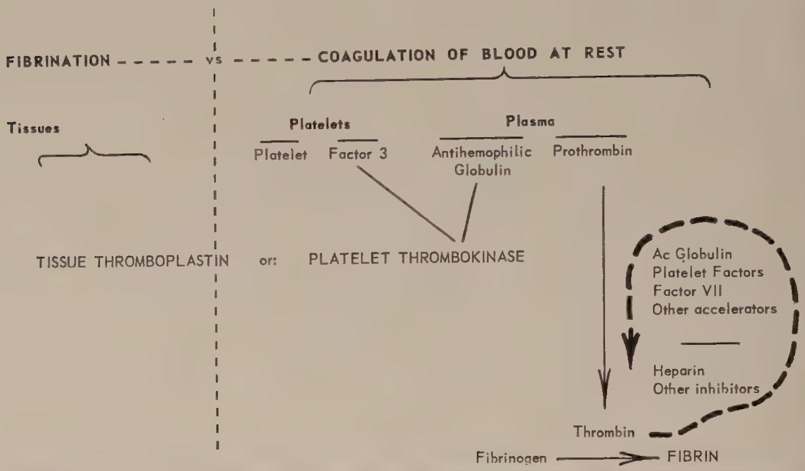

FIGURE 12. Interrelationships of some well-known and contrasting component mechanisms of coagulation. Modified after Seegers,[91] Quick,[57] Steffanini,[97] and others.

Contributions of three sources, the tissues, the platelets, and the blood plasma, to coagulation of fibrin. Interrelated and/or interdependent relationships are possible because active factors from one source may complement or substitute for a factor, with comparable activity or function, from another of the three underlying sources.

Thus, the conversion of prothrombin to thrombin may be initiated either by tissue thromboplastin or by an equivalent—here called platelet thrombokinase—that is known to develop in a primary reaction between a factor (platelet factor 3) residing on particles from the platelets, and the antihemophilic globulin from the plasma. This reaction requires time, and a lag-phase results before initiation of coagulation (see FIGURE 18).

The thrombin, generated by the activation, in turn contributes to further autocatalytic activation of more thrombin (indicated by the broken-line arrow) and to autocatalytic activation of some of the accelerators. The maximal rate of this autocatalytic phase is the same, whether the activation is by tissue thromboplastin or by platelet thrombokinase (see FIGURE 18).

Activities equivalent to those of some of the accelerators from the plasma are available from accelerators contained in the platelets.

Finally, coagulation of fibrinogen from the plasma and thrombosis of platelets (not indicated in this diagram) may each contribute to hemostasis and, within considerable limits, can substitute for each other. A striking example is the relative freedom from a hemorrhagic diathesis in congenital afibrinogenemia—platelet thrombosis must serve these individuals for hemostasis in the absence of plasma fibrinogen. Moreover, thrombin is probably a mediator for thrombosis of platelets as well as for coagulation of fibrinogen.

Of course, additional complexities of additional components, their combinations and their second order—autocatalytic effects, not listed here, may also come into play.

Providently, platelet thrombokinase cannot cause fibrination. Only tissue thromboplastin can cause fibrination defibrination; the physiological locus for tissue thromboplastin to cause coagulation is outside the circulation.

such as can be induced deliberately in experimental animals,[71, 72] there is no formation of a whole-blood clot within the blood vessels; instead, there are characteristic deposits of fibrin from the blood;[72, 73, 84] the blood itself tends to remain liquid, becomes fibrinopenic, and continues to circulate.[50, 73, 83]

Indeed, during fibrination, the plasma of the circulating blood may be converted largely to serum.[83] As during any coagulation, several components of the coagulation and of the thrombus-forming mechanisms may be used up (TABLE 2). It will be noted from FIGURE 14 that the depletion by fibrination

TABLE 2*

Some effects of fibrination	$\xrightarrow{\text{Thrombin}}$	Serum
Fibrination defibrination:	Plasma fibrinogen → fibrin	Fibrinogenopenia
Acquired thrombocytopenia:	Platelets → labilization, lysis	Thrombocytopenia
"Labile factor" deficiency:	Labile factor → active AcG	AcG inactivation
Hypoprothrombinemia:	Prothrombin → Thrombin	Thrombin, inactive
Antithrombin activity:		Antithrombin, increased
Antihemophilic globulin:		AHG, decreased
Hemolysis of erythrocytes:		Hemoglobinemia
"Stable factor" activation:	Stable factor → active factor VII	Active factor VII
Fibrination defibrination		Hemorrhagic diathesis

Some effects of fibrination upon the blood. These are mediated largely by thrombin. Thrombin can be activated in the circulating blood by tissue thromboplastin or, in the laboratory, can be prepared and injected directly. The final effects approach conversion of the circulating plasma to serum. Thus, when fibrination defibrination approaches completion, the circulating "blood" approaches the composition of a suspension of erythrocytes in serum. During fibrination, just as during agitation of a test-tube blood clot, some lysis of erythrocytes occurs. Gradations amounting to variable mixtures of plasma with serum occur in complications of late pregnancy.[83]

* Compiled from various sources, including Johnson,[33] Leary,[42] Lewis,[44] Page,[50] and Soulier.[95]

is different than would be disappearance of coagulation components by inadequate production because, in some ways, the coagulation mechanism remains activated; for example, the stable factor (Factor VII) becomes activated and is stable in serum. Consistent with this, when fibrinogen alone is restored to a defibrinated animal, the support of coagulation is remarkably good.[20, 87]

In fibrination defibrination, the depletion of coagulation and of thrombus-forming mechanisms is sometimes so severe that a hemorrhagic tendency results.[49, 50] Both aspects of the continuous process, fibrination and defibrination, produce several characteristic secondary disorders: the first are based on coagulation, with circulatory obstruction, intravascularly; the second on inadequacy of further hemostasis either by coagulation or by platelet thrombosis. Together, these diverse disorders make up the fibrination-defibrination syndrome.

Outstanding examples may develop in abruptio placentae or, more rarely, in delivery of a hydatid mole, in which fibrination with formation of the most exquisite and characteristic fibrin occlusions may occur.[47, 81] The occlusions may cause circulatory failure, which may lead to obstetric shock even unto death; or, if the hazard of this obstetric shock be survived, as it usually is, this may be followed by a hemorrhagic diathesis with hemorrhage at or near the time of delivery, and hemorrhagic shock, even unto death.

Clinical Complications

Such maternal morbidity or mortality from abruptio placentae as continues to occur is more likely to be the consequence of some "incubated lesion" caused either by the circulatory failure of fibrination or by the hemorrhagic shock: with improving obstetric care, morbidity and mortality seem to be progressively less likely to be caused directly by either the circulatory failure or the hemorrhagic diathesis.[85] This assumes active obstetric management, and further assumes that infection and hemorrhage are adequately managed with blood transfusions and antibiotics. Among these sequelae[47, 81, 85, 95] are the lower nephron syndrome, bilateral renal cortical necrosis, anterior pituitary necrosis with development of Sheehan's syndrome, and adrenal and other hemorrhages; prominently, these include central nervous system hemorrhages either multiple or localized, as in pontine hemorrhage.

Antithromboplastin

Tissue extract has long been investigated in terms of a toxin activity.[45] The toxin is of course tissue-thromboplastin.[71] The reactions to it are different than to most toxins inasmuch as, within a few minutes after a large dose or doses, the test animal becomes relatively or absolutely refractory to further doses of the toxin.[69]

An antisubstance, discovered by Obata, occurs in the blood; this is an antithromboplastin.[71] The relationship of inactivation between tissue thromboplastin and the antithromboplastin is logarithmic, and requires a definite time interval.[68] During pregnancy in the human and in animals the concentration of circulating antithromboplastin increases threefold.[70] Curiously, this does not serve to protect the individual from the immediate toxic activity of tissue-thromboplastin; instead, pregnant animals are some two to three times as sensitive to rapidly injected doses of tissue thromboplastin as are nonpregnant animals.[70, 72, 108]

Mechanism of "Shock of Fibrination" (Shock of Acute Cor Pulmonale)

Naturally, the fibrin deposits of fibrination are more prone to occlude the pulmonary circulation than other circulations.

Other organs which tend to be affected selectively by fibrin deposition are the liver, kidney, and anterior pituitary gland.[47, 67, 81, 93] Repeated episodes seem to predispose to central nervous system hemorrhages;[108] perhaps this is because of some focal intravascular damage due either to an initial fibrin occlusion or to the focal damage from circulatory failure during the initial episode.

Experimentally, obstruction of the pulmonary circulation by fibrination may lead to immediate circulatory failure[71, 75] and hyperacutely to syncope. In abruptio placentae, fibrination is rarely so severe as to cause immediate death; it is possible that fibrination leads to transient, pulmonary hypertension and acute cor pulmonale more often than is recognized.[75] To the degree to which there may be prolongation of pulmonary circulatory embarrassment or of right heart dilatation there may develop an extraordinary kind of shock—the shock of acute cor pulmonale.[6, 90] It is possible that this may lead to the "shock out of proportion to hemorrhage" so long referred to, and so often denied, in abruptio placentae.

Anomalously, measurement of peripheral blood pressure may provide a poor measure of this shock of acute cor pulmonale, especially if caused by fibrination.[90] More subtle observations than those furnished by the sphygmomanometer may be required. Special techniques, including pulmonary arterial

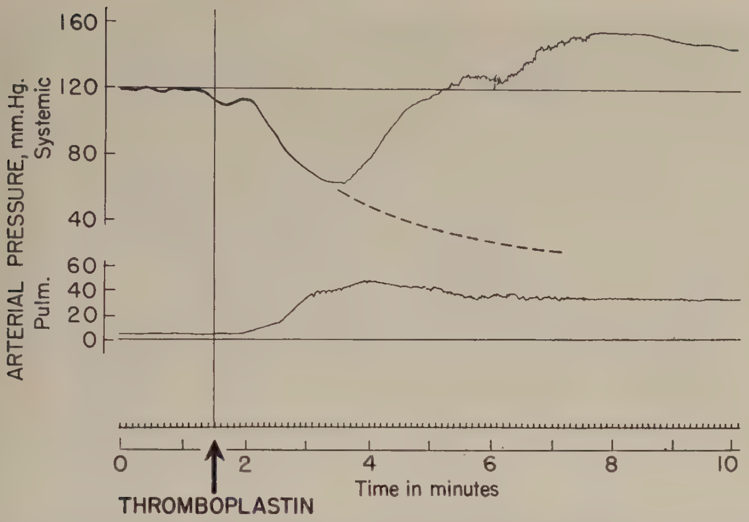

FIGURE 13. Blood pressure changes as recorded through pulmonary arterial and aortic catheters following injection of a nearly lethal dose of tissue thromboplastin intravenously into a dog. Within 30 seconds the pulmonary arterial pressure begins to increase, at first slowly, then more rapidly, approaching a maximum of 50 mm. of mercury. Within 2 minutes the systemic pressure falls as mirror image of the acute pulmonary hypertension to a minimum of 60 mm. of mercury. Thereafter the systemic pressure in this animal recovered remarkably, with restoration above the initial level of 120 mm. despite continued pulmonary hypertension.

The usual course of the systemic pressure following a lethal dose of tissue thromboplastin is indicated by the broken line. From Schneider and Engstrom.[90]

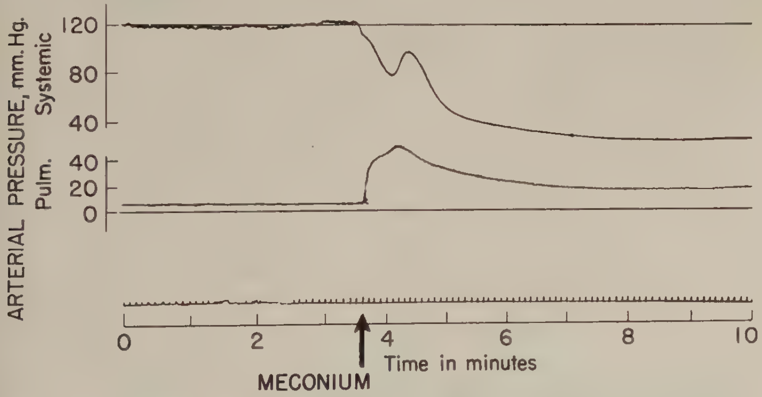

FIGURE 14. Pulmonary and systemic arterial blood pressure changes after intravenous injection of a lethal dose of meconium suspension. The pulmonary arterial pressure rises at once, approaching a maximum of 50 mm. of mercury, and the aortic pressure falls to shock levels. From Schneider and Engstrom.[90]

catheterization used to monitor the blood pressures in experimental animals, as in FIGURES 13 and 14, may be deemed not properly applicable in human obstetric shock, even if facilities for cardiac catheterization and time are available during the emergency conditions.

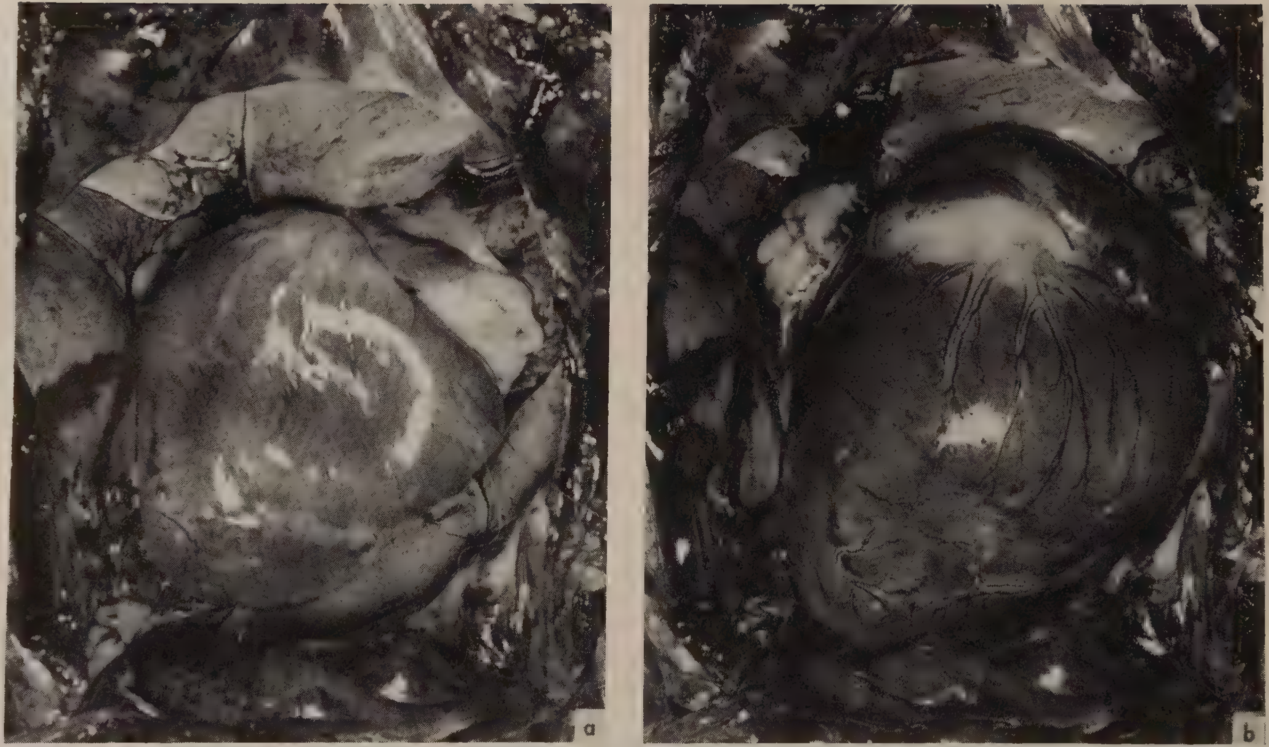

FIGURE 15. (*a*) Dog heart seen during systole with pericardium opened, before injection of placental tissue thromboplastin. The right ventricle is emptying. The normal, right atrial appendage is barely visible behind a lobe of lung. The myocardium has the healthy, light color of good oxygenation. (*b*) The same dog heart after injection of a lethal dose of placental tissue thromboplastin. There is acute dilatation of the right heart with cyanosis of the myocardium, with marked distension and engorgement of the coronary vessels. The right atrium is engorged and extends over the entire base of the heart, adding to the right axis deviation and overriding a lobe of lung previously visible above the right ventricle.

As shown in FIGURE 13, despite a tendency for the increased, pulmonary hypertension to continue during fibrination in the dog, the systemic blood pressure can be restored quickly and maintained spontaneously.[90] It should be added that the arteries of the extremities may transiently be essentially pulseless; if the femoral artery has been previously exposed, it may be observed to become constricted markedly during onset of fibrination. The conjunctivae are pale. The tongue is dusky, cyanotic. The liver becomes palpably engorged and tense. At direct observation the right heart becomes dilated. Probably the cardiac output is greatly diminished, at least transiently. The acute cor pulmonale develops hyperacutely and, if it is to be relieved, is relieved almost as rapidly. The sudden return of the myocardium from its dusky cyanosis to a healthy red is as dramatic as is the sudden reduction of the dilated right ventricle, with return from arrhythmias to effective contractions of the heart; these restorations (compare FIGURES 15*a* and *b*) are as dramatic as restoration of the systemic blood pressure shown in FIGURE 13, to which they correspond.

The animal either soon dies or soon recovers from its syncope and/or convulsions. The duration of the severe circulatory failure is a few minutes at most. Rarely does the animal remain in prolonged syncope from fibrination—rarely does this persist long enough for a state of secondary shock to develop, although this does occur at times. The systemic hypotension may be of such brief duration that in the absence of a continuous recording, as in FIGURE 13, one might wonder if hypotension had occurred at all; if the blood pressure of the dog had been measured with a conventional sphygmomanometer during the brief interval of severe hypotension, only to reveal the pressure at normal levels a moment later, one might wonder about the validity of such an observation—hence the value of the continuous recording.

Vasopressor Action

The early restoration and maintenance of systemic pressure, despite continued observations of some degree of acute cor pulmonale, are suggestive of a diminished cardiac output, and seem to be consistent with marked, systemic vasoconstriction.[89, 90] A vast activity of serotonin may be released from the platelets during fibrination, and it is not impossible that this may persist sufficiently to serve as a chemical mediator to support a vasopressor action. Because of some degree of similar effect on the lesser circulation, serotonin may be of significance also as a chemical mediator in the mechanism of production of the acute cor pulmonale itself.[7] Large doses of serotonin can cause renal cortical necrosis in rats.[51]

Acute Cor Pulmonale

Acute cor pulmonale is a clinical term. In the acute cor pulmonale of fibrination, acute dilatation of the right heart is caused by acute pulmonary hypertension. The hypertension is due to acute obstruction to the pulmonary circulation. For special reasons, interested investigators may object to use of the term acute cor pulmonale or may place restrictions upon its application. The pathologist may prefer the term acute dilatation of the right heart, a finding that sometimes (but not always) can be demonstrated objectively at autopsy.[94]

The internist may wish to define the disorder as "acute pulmonary hypertension" caused by a pulmonary disorder extrinsic to the pulmonary vessels, but of course having an occluding effect, reflexly or otherwise, upon the pulmonary vascular tree. An example is the hyperacute dilatation of the right heart, secondary to the "anaphylactoid" reaction, that can be caused by injection of diverse materials into the circulation, or that Steiner could cause in animals by inhalation of benzene vapor.[98] The irritant caused contraction of the smooth muscle of the bronchioles. Likewise, the irritant causes contraction of the smooth muscle of the pulmonary arterioles. Steiner knew, by comparison with the work of Hanzlik and Karsner,[25] that a comparable "anaphylactoid reaction" with acute dilatation of the right heart could be caused by introduction of various kinds of particulate debris into the circulation; perhaps some common pathway of pulmonary vasopressor activity can be invoked.

As in the more general interpretation of the internist, we use the term acute cor pulmonale to mean acute dilatation of the right heart due to acute pulmonary hypertension, in turn due to pulmonary vascular obstruction; for the present studies the obstruction would be of origin intrinsic to the pulmonary vessels themselves.[84] In this sense the acute or, indeed, hyperacute cor pulmonale that can be induced by fibrination is primarily the result of intravascular occlusion of the pulmonary vascular bed. We find the term acute cor pulmonale to be useful whether the occlusion arises from meconium embolism, an experimental counterpart of amniotic embolism, the occlusions arising on the basis of disseminated embolic debris, perhaps with inhibition of coagulation, or on the basis of fibrin deposits built-up *in situ* to form intravascular occlusions, as in the fibrination of thromboplastin injection or of abruptio placentae. Perhaps the pulmonary vascular obstructions are reinforced by reflex mechanisms of vasospasm and perhaps these or additional reflexes also produce bronchiolar constriction. Primary, however, are the vascular obstruction of fibrin on the one hand,[73] and of debris on the other hand,[99] either of which can be demonstrated microscopically.

This interpretation of acute cor pulmonale is comparable to the classic example of acute cor pulmonale, which results not from pulmonary disease external to the pulmonary vessels, but from gross pulmonary embolism either of deported thrombi, or gross emboli from "bland" coagulation; the results and analyses of innumerable studies are available for consideration.[12] Suffice it to say that the acute cor pulmonale of gross embolism can be and has been studied clinically from: (1) physical findings of accentuated pulmonary heart sounds and enlargement of the area of cardiac dullness and development of pulmonary edema; (2) X-ray evidence of enlargement of the heart and of diffuse pulmonary edema; (3) electrocardiographic changes of pattern and rhythm characteristic of acute dilatation of the right heart with right axis rotation and myocardial anoxia;[6] and (4) postmortem findings, if the duration of disorder be long enough for pulmonary edema and acute dilatation of the right heart; the duration, of course, is not sufficient for hypertrophy of the right heart.

The data of Gibbon and Churchill[22] are particularly significant as regards subtle change.[12] These investigators distinguished between acute cor pulmonale produced by embolic or other experimental occlusions of the main pulmonary artery and occlusion of the pulmonary branches. With occlusion of the main artery, there is pallor and syncope; with occlusion of the branches, cyanosis.

These data are plotted into graphic form in FIGURES 16 and 17.

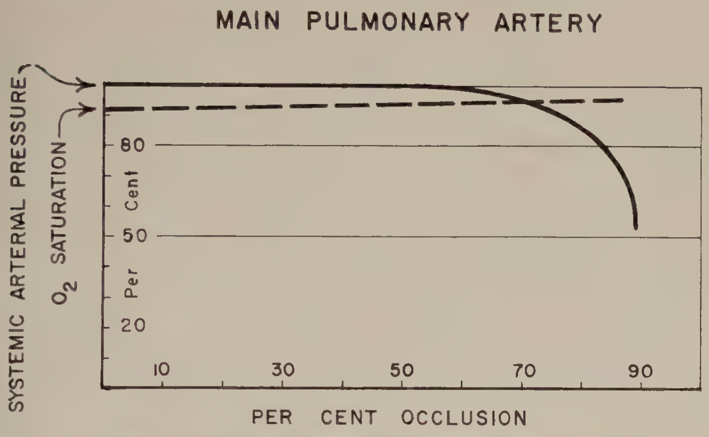

FIGURE 16. Systemic arterial blood pressures and oxygen saturation of the arterial blood after increasing degrees of occlusion of the main pulmonary artery. The systemic pressure begins to decrease after 60 per cent occlusion. Paradoxically, the percentage of oxygen saturation tends to increase even when the blood pressure approaches shock levels.

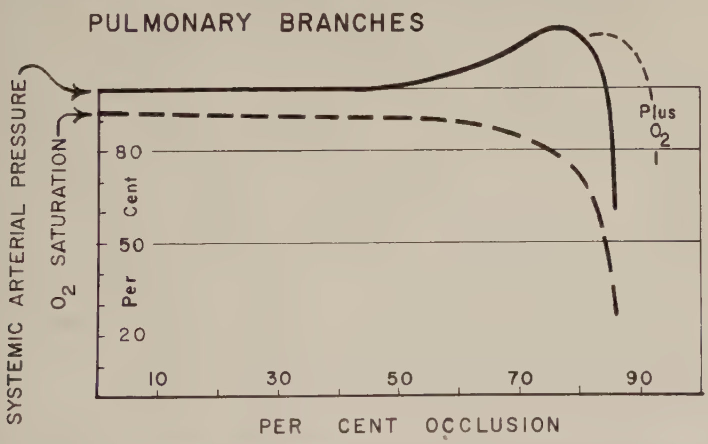

FIGURE 17. Blood pressures and oxygen saturation after increasing percentage of occlusions of the pulmonary arterial branches. Beyond 60 per cent occlusion the somewhat variable arterial pressures tended on the whole to increase somewhat before final collapse; the oxygen saturation gradually decreased with increasing percentage of occlusion, falling to critical levels beyond 70 per cent occlusion. If oxygen was administered artificially, both oxygen saturation and systemic blood pressures were well maintained until 90 per cent occlusion was reached. FIGURES 16 and 17 are plotted graphically from the data of Gibbon and Churchill.[22]

Paradoxically, with increasing degrees of occlusion of the pulmonary artery itself there was a slightly increased oxygen saturation of the arterial blood; the increased saturation continued up to the very degree of occlusion beyond which the systemic circulation became imperceptible and beyond which the individual lapsed into syncope.[22] Therapeutic administration of oxygen was of no benefit.

By contrast, with increasing percentage occlusion of the branches of the pulmonary arterial tree there was, beyond a critical point, gradually decreasing oxygen saturation of the arterial blood.[22] This was either without change of systemic arterial pressure or with variable changes, there being a tendency for transient increases of arterial pressure until a degree of occlusion was reached beyond which there was final collapse.

With occlusion of the pulmonary branches, if oxygen was administered, the systemic blood pressure was maintained until a still greater percentage of the vascular bed was occluded (FIGURE 17); the individuals were able to survive to a higher percentage of total pulmonary vascular occlusion.

Although parallel studies for occlusions farther out into the vascular bed, that is, occlusions into a higher order of branchings, are not known to be available, knowledge of the benefits from administration of oxygen may well be pertinent to the management of sudden human obstetric shock of acute cor pulmonale (whether the obstetric shock be due to abruptio placentae or to amniotic embolism). It is natural to provide oxygen by artificial respiration, under pressure if need be, to a mother suddenly and unexplainably cyanotic and in collapse, as may be the situation when amniotic embolism occurs acutely. In a few instances mothers have been reported to survive severe amniotic fluid embolism temporarily or permanently with oxygen serving as the chief therapeutic agent of value. Detailed recordings seem to be lacking concerning the possible benefits of oxygen therapy in the obstetric shock out of proportion to hemorrhage of abruptio placentae. Perhaps this disparity, is due to the possibility that the obstetric shock of abruptio placentae may occur before admission to the hospital or before transfer to the delivery suite where anesthesia apparatus is available for artificial respiration. Also, it is possible that in amniotic embolism the shock may be more severe because here the acute cor pulmonale may tend to be more persistent, while that of abruptio placentae tends to be transient.

It is noteworthy in the studies of Gibbon and Churchill that with their occlusions of the "pulmonary vascular bed" (that is, their occlusion of the arterial branches) the systemic arterial pressure is not only maintained, but may even be variably increased in the range of onset of inadequate oxygenation of the blood. Thus, particularly with occlusion of branches, observation of the systemic blood pressure provides little or no warning until the moment of final collapse. This is pertinent to observation of mothers with obstetric shock of amniotic embolism or of abruptio placentae, for it implies that the chief measure of shock upon which the obstetrician and the internist alike have learned to rely, namely the systemic blood pressure, may remain essentially uneffected until the final moment of the patient's collapse.

It is perhaps for this reason that the "obstetric shock" of amniotic embolism, so clearly predicated by Steiner and Lushbaugh on the basis of pathological and experimental observations, is so subtle that obstetricians often fail to observe it. The number of recorded cases with blood pressures of shock levels for considerable intervals gradually grows, yet they still seem to be too infrequent to influence statistics of maternal mortality.[100] To deny that the patient may have been in a state of shock because she had a relatively normal blood pressure until the moment of final and sudden collapse does not serve to restore her to life.

Clearly, if obstetric shock is defined in terms of falling blood pressure it may not be always possible to detect this kind of shock of acute cor pulmonale. To define shock in terms of a predetermined degree of fall in blood pressure may be to exclude the shock of amniotic embolism or of abruptio placentae, by definition only. The patient may nevertheless suffer syncope or death without the benefit of diagnosis.

Defibrination Versus Acutely Acquired Thrombocytopenia: Panhemostatic Failure

To the degree to which fibrinopenia develops, the circulating blood may be deprived of its capability again to support coagulation. Probably both a potential hemorrhagic diathesis due to fibrinopenia and varying degrees and kinds of hemorrhagic diatheses may develop; the exact nature of these may depend upon variations in the degree of depletion of one or more of several coagulation and hemostatic mechanisms. There is a panhematologic depletion.[83]

Platelets are depleted during fibrination[11] and, upon occasion, thrombocytopenia seems to have contributed as much, or more, to a hemorrhagic diathesis than fibrinopenia; in one patient[27] the bleeding from minor traumata of the obstetric delivery and from the regions of vena puncture points was still increasing and the bleeding tendency correlated better with the platelet count, which was still gradually decreasing, than with the plasma fibrinogen, which was already gradually increasing. Doubtless if a severe degree of this kind of fibrination-defibrination had been first investigated by a cytohematologist, he would have made a diagnosis of "acutely acquired thrombocytopenia."[83] As an extension of this outlook, perhaps study of acutely acquired capillary fragility would also be of interest.

In addition to the fibrinogen-fibrin coagulation mechanism of the plasma there are accessory, interdependent mechanisms for hemostasis, that can act together with, or in the absence of, plasma fibrinogen, to maintain or to restore the integrity of the vascular bed. Not least among these other mechanisms may be functions of the platelets, including platelet agglutination, platelet thrombosis, and action of serotonin, the platelet vasoconstrictor. Indeed, in certain more primitive organisms, a primitive form of thrombocyte alone, without plasma fibrinogen, is adequate for the lesser demands of hemostasis of a less dynamic circulation.

Incidentally, it may well be that, quite aside from plasma afibrinogenemia, platelets themselves may contain a "platelet fibrinogen"; from the data[2, 41] it may be wondered whether, in the human, a small amount of a protein that ca be coagulated by thrombin appears in relation to the platelets themselves.

The question may be asked whether there is a kind of platelet fibrinogen such a possibility may be significant for platelet thrombus formation in genera

By comparison, in studying thoroughly washed bovine platelets, a protei component could be extracted that coagulates under the influence of thrombin.[59] This platelet fibrinogen was of a chemical composition indistinguishable from that of plasma fibrinogen.

In summary, defibrination is merely a convenient term for a process causin profound depletion of many components of the hemostatic mechanisms coagulation, thrombosis, and vasoconstriction. To the degree to which defibrination can be measured, it can serve as an approximate index of this panhemostatic depletion of fibrination-defibrination. When and if thrombocytopeni develops disproportionately, this may be the most significant factor in a acutely acquired hemorrhagic diathesis.

Blood Coagulation Mechanism: Tissue Thromboplastin Versus Platelet Thrombokinase

Because fibrination, as distinguished from simple test-tube blood coagulation can be initiated by tissue thromboplastin but not by platelet thrombokinase it becomes necessary to distinguish the two initiators, if not at the chemical level at least at the biological level, or at both (FIGURE 12).

In the blood coagulation mechanism of Morowitz, an initiator of coagulation can activate conversion of prothrombin to thrombin. In his second stage of coagulation, thrombin converts fibrinogen to fibrin. In an appropriate environment, the fibrin may then coagulate into a fibrin clot.

The initiator of coagulation of Morowitz was called thrombokinase. As is well known, thrombokinase activity can be generated in the plasma in a preliminary stage of coagulation in the presence of platelets (FIGURE 12). Another apparently already active initiator from tissues was called thromboplastin. By general consent and usage, European and American investigators have gradually adopted the term thromboplastin for both initiators, although these can hardly be considered identical.[85]

Meanwhile, as the result of the studies that led to the term fibrination,[11] occasion to distinguish platelet thrombokinase from tissue thromboplastin again arises.[83]

In formulating the problem it is noted that platelet thrombokinase, while marvelously adapted to initiate the blood coagulation of physiologic hemostasis, is nevertheless unable to initiate fibrination.[89] Were this not so, there would be an ever-present hazard that any thrombus, however innocent, might tend to initiate, or might tend to propagate, fibrination to completion with the attendant pathological implications of fibrination defibrination.[83] Whether or not initiation of fibrination by platelets may be found in the future, platelets have not yet been observed to activate fibrination.

Its lag phase may be a factor of safety by which the platelet-thrombokinase mechanism prevents extension of its activity to fibrination before significant

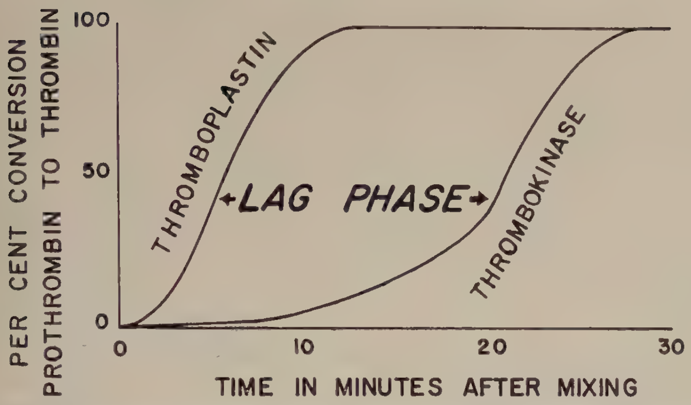

Figure 18. Lag phase before activation of prothrombin to thrombin by tissue thromboplastin and by platelet thrombokinase in a purified system. Thromboplastin begins to initiate a conversion of prothrombin to thrombin almost at once. Thrombokinase has a long lag phase, after which it activates prothrombin equally completely and at an equally rapid rate. From Schneider,[83] as modified from Johnson *et al.*[34]

initiation of the first stage of coagulation. Demonstration of this lag phase before conversion of prothrombin to thrombin by platelet thrombokinase, as compared with essentially no lag phase for tissue thromboplastin, is shown in FIGURE 18.

This lag phase doubtless has significance for the circulating blood. Conversely, this lag phase of the platelet-thrombokinase mechanism has little significance for the initiation of coagulation if the blood is in a container outside the body, within a thrombus, or in stasis, for the platelet particles do not then become removed from the field of reaction by phagocytosis; there is then, little or no premium on rate of onset for thrombin formation, that is, no premium for immediate initiation of coagulation.

This is not to imply that either platelet thrombokinase or tissue thromboplastin is a less potent initiator of coagulation than the other, each after its own lag phase. From FIGURE 18 it is clear that, once the autocatalytic phase of coagulation is reached, both are equally potent: both convert prothrombin to thrombin at the same maximal rate and to the same final degree. Because platelet thrombokinase and tissue thromboplastin are of equal potency it cannot be said that low potency prevents platelet thrombokinase from causing ibrination.

In summary, in the circulating blood the lag phase for platelet thrombokinase may play a key role in permitting removal of platelet particles by phagocytosis before initiation of fibrination; that is, it may permit removal of the particles without fibrination.

Phagocytosis Versus Platelet Thrombokinase

Conceivably, in the circulation the platelet particles contributing to the platelet-thrombokinase mechanism of coagulation may become phagocytized

TABLE 3
EXPERIMENTAL EMBOLISM OF MECONIUM SUSPENSION
Effects upon Coagulation of the Blood of the Dog

Dose of meconium suspension:	None	Small	Large	Overwhelming
Clinical change	None	Mild	Syncope*	Prompt death†
Shock	No	±	++++	No
Prolongation of coagulation time:				
Lee White (minutes)	0	0	++++	±
Quick test (seconds)	0	0	++++	0
Plasma components:				
Fibrinogen	Unchanged			
Prothrombin	Unchanged			
Ac-globulin	Unchanged			
Thrombocytopenia	0	±	++++	++
Protamine titration	0	±	++++	0
Heparin assay	0	±	++++	0
Inhibition of coagulation, mixing with normal blood	0	0	++++	0

* Syncope: comparable to the "obstetric shock" of amniotic embolism of Steiner an Lushbaugh.

† Prompt death: death from circulatory failure so promptly that there was essentiall no interval of shock.

in an interval as short as, or shorter than, the lag phase before activation by platelet thrombokinase. As in the observations of Krogh[40] or of Knisely *et al.*,[3 any particle of foreign matter introduced into the blood stream is immediately coated with a layer of protein, even before the droplet containing it becomes detached from the micropipette through which it is introduced. Thereafter, within the first passage through the circulation and upon the first chance contact with the endothelium of a vascular bed capable of phagocytosis, the coated mass becomes at once adherent to the endothelium. The mass is then whipped against the endothelium by the flow of the blood stream itself; the protein-encased foreign particle then becomes engulfed and removed from the circulation.[38] Thus an appreciable lag phase, such as that observed in TABLE 3 during the initiation of coagulation by platelet thrombokinase, if representative of a similar lag phase in the circulating blood, may largely permit removal of platelet fragments from the field of reaction before these can initiate fibrination.

Perhaps not of least significance for the present considerations is the phagocytic activity of the pulmonary vascular bed.

Phagocytosis Versus Tissue Thromboplastin

Tissue thromboplastin is of macromolecular size or is attached to particles of macromolecular size and, conceivably, may become phagocytized in some way also, either directly or with fibrin that is formed. The observations of Page *et al.*[52] indicate that survival in the circulation is short-lived; it probably survives no more than a few times through the circulation.

Whatever the survival by comparison with that of platelet particles, the survival time of tissue thromboplastin in the circulating blood must be greater than its own lag-phase time because the observations are that extracts, potent in tissue thromboplastin, do cause fibrination defibrination.

Fibrination: Fibrinopenia Versus Afibrinogenemia

It is pertinent that the progression of fibrination defibrination is to fibrinopenia and rarely, in our clinical or laboratory experience, or in the laboratory experience of others, to afibrinogenemia; that is, the usual result of fibrination is partial, not complete, defibrination. Phillips *et al.*[56] found that some of their markedly fibrinopenic patients with abruptio placentae had no fibrinogen; during this acute and extreme phase they also found increased fibrinolytic activity.

There is evidence that during progressive fibrination defibrination the process may be retarded by release, or by autoactivation, of one or more inhibitors of the first or of the second stages of coagulation; this can be detected as a "thrombin-inhibitory" activity.[8, 60, 61]

While occurrence of some kind of a low-grade inhibitory activity can be confirmed in venous blood, drawn after fibrination,[60] the inhibitor could not be identified specifically as heparin or as antithrombin.[82] Heparin is released during the shock of comparable experimental meconium embolism.[82] Whatever the nature of the inhibitors that may be released or activated by fibrination, some mechanism may tend to limit defibrination, short of afibrinogenemia.

Extraordinary measures are required to produce sufficiently complete fibrination to cause complete incoagulability. To bring about the final stages of afibrinogenemia, Fulton and Page[20] in 1947 found it expedient to inject purified thrombin intravascularly. By careful infusion Jürgens and Studer were able to bring about defibrination with purified thrombin alone.[35]

Concurrence of Abruptio Placentae and Amniotic Embolism

Abruptio placentae and amniotic embolism are rare disorders (severe reactions, approximately 1:1000 and 1:8000 deliveries, respectively). These disorders tend to occur under partially similar circumstances. Hence their concurrence is of interest.

TABLE 4*

THROMBOPLASTIN YIELDS IN, OR CONVERTED TO, MOUSE UNITS PER GRAM OF SOURCE MATERIAL IN SIMPLE SALINE EXTRACT

Urine	±	Placenta	
Amniotic fluid	1	First trimester	2,000
Liver	10	Term	200
Skeletal muscle	20	Decidua	
Kidney	50	First trimester	
Brain	50	Curettings (D. & C.)	2,000
Lung	200	Uterus (hysterectomy, ectopic pregnancy)	2,000
Platelets	None	Term	
		Uterus (curettings, at cesarean section)	2,000
		Placenta, scrapings (basal plate)	1,500
		Membrane, scrapings	20

* Modified after Schneider.[73]

Abruptio placentae and amniotic embolism, both of major proportions, hav already been proved in at least one patient[106] whose blood became totally incoagulable; it would not clot spontaneously.

On the basis of present knowledge, total incoagulability would hardly be expected from fibrination defibrination of abruptio placentae. In their report some investigators discussed afibrinogenemia[106] and considered that this had developed as a result of coagulation caused by the patient's amniotic embolism. In view of the low content of tissue thromboplastin in amniotic fluid (TABLE 4 there would seem to be little probability that amniotic embolism would have caused significant or complete fibrination defibrination.

Correlation of further data from the original protocol[85] with data from experimental animal meconium embolism[82] leads to quite another interpretation both as to the nature of the incoagulability and of its cause, namely that a potent inhibitor of coagulation was released within the body because of the obstetric shock of the amniotic embolism.[85]

Amniotic Fluid Embolism

Incoagulability of drawn, venous blood does not necessarily provide an adequate basis from which to conclude that there is afibrinogenemia.

Van Sante reported total incoagulability of drawn blood of a patient with obstetric shock of amniotic embolism.[105] He found that the plasma fibrinogen level was normal and that there was a potent inhibitor. His patient survived. His clinical diagnosis is well substantiated.[105]

Another patient whose blood became totally incoagulable was found by her prosector in 1940 to have in her postmortem blood a normal fibrinogen level of 250 mg. per cent; a potent inhibitor of coagulation was present.[85] Abruptio placentae had been demonstrated at cesarean section.[106] The diagnosis of amniotic embolism was established at postmortem examination.[106]

This patient and the surviving patient of van Sante had unquestionable, clinical, obstetric shock of the kind implied by Steiner and Lushbaugh at the time of their original discovery in 1941, which they later reaffirmed and emphasized.[100] This "obstetric shock of amniotic embolism" is distinct from shock of hemorrhage. Hemorrhagic shock may also occur or may become superimposed on the obstetric shock. This kind of shock is of significance both as a part of the amniotic embolism syndrome and of the fibrination-defibrination syndrome (FIGURES 13 and 14).

The fact that complete incoagulability, without defibrination but with a potent inhibitor of coagulation in the circulating blood, has been observed in a patient with a postmortem diagnosis of amniotic embolism is reminiscent of the findings of total incoagulability, yet with normal fibrinogen, in dogs following experimental meconium embolism.[80, 82]

This observed effect of inhibition of coagulation following clinically recognizable obstetric shock of amniotic embolism is indicated diagrammatically in FIGURE 19 (compare with FIGURE 2). In the patient with both abruptio placentae and amniotic embolism[101] there may have been a stage in which a tendency for fibrination by abruptio may have become inhibited by the inhibitor that is released in obstetric shock of amniotic embolism.[85]

Meconium Embolism in the Dog: Release of Heparin

Meconium embolism is the laboratory counterpart of amniotic embolism. As indicated in TABLE 3, the circulating blood becomes incoagulable when, and only when, the animal undergoes shock as a result of this embolism. If the dose is too small to cause shock, there is no effect upon components of coagulation, save possibly depletion of platelets. Likewise, if the dose of meconium is so overwhelming as to cause syncope and death so promptly as to leave no appreciable, intervening interval of shock, there is no effect upon coagulation other than, possibly, depletion of platelets.

It is unlikely, therefore, that the incoagulability of the blood results from an inhibitor carried in the meconium or is the result of release of an inhibitor because of a chemical mediator in the meconium.

Only those dogs with doses of meconium suspension adequate to cause syncope and shock, but not so large as to cause death without an interval of shock before death, developed totally incoagulable blood (TABLE 3). The plasma fibrinogen remained normal. A potent inhibitor appeared in the blood. Analysis by F. C. Monkhouse of the University of Toronto, Toronto, Canada, proved that the inhibitor was heparin.[82]

In summary: heparin was released as a result of the shock of acute cor pulmonale caused by meconium embolism.[82]

Monkhouse cautions that heparin has never been demonstrated from human sources. Thus far, blood of a patient with obstetric shock of amniotic embolism has not become available to him for specific analysis for heparin.

Obstetric Shock of Amniotic Embolism

In studying the physiology of abruptio placentae, it becomes obvious that the shock out of proportion to hemorrhage occasionally encountered in practice or occasionally described in the literature may be truly obstetric shock of abruptio placentae.[81] A clinical control study is needed. Such a control seems to be provided exquisitely by the obstetric shock of amniotic embolism of Steiner and Lushbaugh. Outstanding in this control disorder is the fact that, quite opposite to fibrination, there may be inhibition of coagulation (TABLE 3 and FIGURE 19). The factor common to these two acute disorders of late pregnancy is that in both there may be hyperacute obstruction of the pulmonary vascular bed. In both there may result shock of acute cor pulmonale.

The mechanism of this kind of shock of acute cor pulmonale has several subtle aspects:

Steiner and Lushbaugh[99] in their original pathological observations and experimental investigations with animals consider not merely the possibility that the mechanism may be one of mechanical occlusion of the pulmonary vascular bed but that, in addition to the embolic debris that can be demonstrated in human amniotic embolism or that can be induced in experimental meconium embolism, the effect of the vascular occlusions conceivable may be further

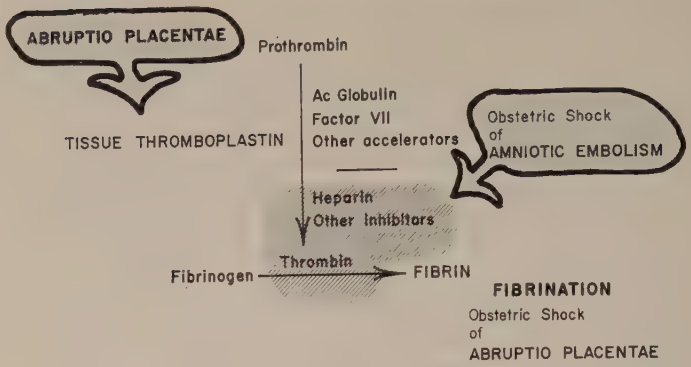

FIGURE 19. Inhibiting effect from shock of amniotic fluid embolism upon blood coagulation and upon fibrination. An inhibitor sufficiently potent to inhibit coagulation of drawn blood in the test tube completely is present during obstetric shock of amniotic embolism. A similar inhibiting effect is present in dog blood during shock of experimental meconium embolism and is identified as heparin. Unusually large amounts of thrombin are required to produce coagulation in the presence of the inhibitor.

potentiated by a physiological reflex vasoconstriction of the pulmonary arterial tree. Whatever the details of the possible reflex mechanisms, the observations of Steiner and Lushbaugh are precise and definitive: (1) minute particulate embolic debris was observed to have lodged within the radicals of the pulmonary vascular bed; (2) pulmonary hypertension must have resulted; (3) in the experimental animals, acute dilatation of the right heart resulted; (4) no fibrin deposits were found; (5) dissolved or suspended proteins of clarified amniotic fluid did not produce an anaphylactoid or an anaphylactic shock; and (6) there was anaphylactoid shock in the sense of Hanzlik and Karsner in which minute particulate embolic debris, of whatever kind,* produced pulmonary hypertension and acute dilatation of the right heart, not merely by particulate occlusion of the pulmonary vascular bed, but in part by activation of pulmonary vascular (and bronchiolar) spasms.

Unfortunately the term anaphylactoid shock was later grossly misquoted as anaphylactic shock. As a result additional investigations have been carried out. Cron *et al.*[10] devised an experiment in which, instead of clarifying the amniotic fluid of its particulate debris by centrifugation or by filtration before injection, they permitted the experimental animals themselves to clarify the injected amniotic fluid of its suspended particulate debris. They injected whole amniotic fluid with its usual debris into the femoral arteries of dogs; no anaphylactic or other shock occurred. As expected, they did find hemorrhagic infarcts peripheral to the injection, consistent with the expected embolic phenomena; there was no embolism to the lung. The animals were not adversely affected. Like Steiner and Lushbaugh, Cron and his co-workers concluded that amniotic fluid does not cause anaphylactic or anaphylactoid shock; they deduced that it is the particulate debris of amniotic fluid that causes the shock of amniotic embolism. Another group of investigators, Hunter *et al.*,[29] avoided the problem of heterologous sensitivities by intravenously infusing amniotic fluid from the conceptuses of two dogs back into the dogs without apparent ill effects. Tio[103] in Peru, intravenously injected clarified amniotic fluid in volumes from 5 to 500 ml. into 73 human patients; he observed no untoward results.

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* Hanslik and Karsner studied the effects of a most heterogeneous group of materials. These included not only suspensions and embolic debris, but dissolved crystalloids and tissue extracts that doubtless contained active thromboplastin. Without differentiating between the causes or mechanisms, they considered that each of these caused some kind of anaphylactoid shock distinct from anaphylactic shock.

While the theoretical possibility must always remain that, in the amniotic fluid, proteins from the fetus incompatible for the mother might permit sensitization and anaphylactic responses in the mother, to the present time there are no observation or other evidence to show that this does indeed occur. The same experiments quoted above[10, 29, 82, 99, 103] would seem to make it improbable that significant fibrination occurs because of infusion of amniotic fluid.

It is worthy of emphasis that Steiner and Lushbaugh were concerned with the peculiar kind of anaphylactoid shock that results from pulmonary embolism of minute particulate debris.

As described above, subsequent investigators confirm and extend the finding that components of amniotic fluid other than its particulate debris are essentially harmless for anaphylactic, anaphylactoid, or fibrination reactions. The possibility that the shock may be due to pulmonary vascular embolism of particulate debris has been investigated more directly by Gross and Brown[24], who injected ultramicroscopic particles intravascularly into rabbits. The more finely divided the foreign particles, the more effective per unit of weight they were. The results were as though the particles became coated with a protein from the plasma, possibly fibrinogen, reminiscent of the studies of Knisely and his co-workers,[38] in which the coated particles then agglutinated and agglomerated to build up proteinacious masses sufficiently large to occlude small vessels. The observations of Gross and Brown were that, after an interval, microscopically visible proteinacious occlusions could be found, particularly in the pulmonary vascular bed in stained tissue sections. The animals developed pulmonary edema and shock.

As with mothers suddenly dead of amniotic embolism, animals into which overwhelming doses of particulate matter are injected and that die almost at once develop minimal clinical or anatomical pathological findings of pulmonary edema. Patients who do not die at once of amniotic embolism may develop increasing signs and symptoms of obstetric shock of amniotic embolism with pulmonary edema, whether they die within a few hours or recover.[92]

In summary, it is possible that some release of tissue thromboplastin and some degree of fibrination defibrination may occur in amniotic embolism. The chief offending material, however, is the particulate debris, and this embarrasses the circulation by disseminated pulmonary embolism, as shown by Steiner and Lushbaugh in 1941. If this leads to severe obstetric shock of amniotic embolism, one result may be release of a sufficiently potent inhibitor to render the blood incoagulable as predicated from animal experiments by Schneider and as found in the human by T. J. van Sante. In experimental meconium embolism, this inhibitor is heparin; in human amniotic embolism the inhibitor is not yet identified.

Abruptio Placentae Versus Abruptio Marginale

Abruptio placentae increasingly becomes a technical term.[73, 76, 77, 81] Abruptio is not to be confused with mere "premature separation of the normally implanted placenta." Abruptio placentae is the retroplacental premature separation of the placenta;[74] of necessity, there is separation in a region of maternal transdecidual, arterial blood supply.[58, 73, 77, 80, 96]

In particular, abruptio placentae is not to be confused with premature marginal separation of the placenta.[18, 19, 64, 73, 74, 77, 81] Abruptio marginale involves venous bleeding only, and no arterial bleeding.[77] Clinically, abruptio marginale is characterized by painless vaginal bleeding. Marginal separation of the placenta has not been recorded as the cause of fibrinopenia; it is unlikely to be a frequent cause.

To include statistics of the frequent and clinically benign abruptio marginale as a kind of abruptio placentae[16] is to increase the obstacles to understanding and to management of abruptio placentae. In his masterful text Eastman[1] is careful not to dilute his statistics; he deals separately with severe abruptio a classification into which his cases of marginal separation seem not to fall. Cesarean section for abruptio placentae, at an incidence that can be calculated to be one in one thousand deliveries (45 abruptios in 40,000 deliveries in 10 years), is little different from the incidence from active obstetric management except perhaps for delay before carrying out that management. As the severity of complications increases with delay before delivery,[53, 73] active obstetric management may ultimately become necessary under increasingly adverse conditions.[53, 74]

The clinical diagnosis of marginal separation is a diagnosis of exclusion—exclusion of placentae previa and of abruptio placentae.[86] Conservative obstetric management for vaginal delivery is then commendable.[18, 19] Management of abruptio is different.

Management of Abruptio Placentae

The first step in the management of abruptio placentae is clinical differential diagnosis. As indicated, it is necessary to distinguish from marginal separation of the placenta. This is usually not difficult if use is made of the cardinal symptoms and signs: uterine pain, tetany, and tenderness. Once the diagnosis is made, the physician is committed to his own course of management, whether it be conservative or active. In some schools of management, it has long been the practice to carry out prompt delivery.[14]

A growing experience of my associate, Robert G. Braden, and of my own is that, whenever vaginal delivery is not already imminent, delivery by cesarean section serves to minimize mortality and morbidity both for mother and for fetus. Lest this seem too drastic an approach, it should be added that in the hospitals where we practice it is not unusual for two hours to pass before it is

TABLE 5

COMPLICATIONS OF ABRUPTIO PLACENTAE WITH DEFIBRINATION*

Case	Hours in hospital before cesarean	Presenting symptoms and signs	Treatment before cesarean	Clinical complications	Hours: abruptio to cesarean or hysterectomy	Delay in hospital, hours
A	44	Pre-eclampsia, severe	Voorhees' bag Stimulation of labor before abruptio	Obstetric shock Eclampsia, postpartum Death after five days	8	8
B	7	Abruptio placentae	Expectant	Obstetric shock Hemorrhagic diathesis Supracervical hysterectomy	9	7
C	9	Abruptio placentae	Expectant	Obstetric shock Hemorrhagic diathesis	12	9
D	10	Abruptio placentae Rh sensitization Dead fetus syndrome	Pitocin stimulation Four hours labor, not progressive	Rupture of uterus, hemorrhage Hemorrhage, post hysterectomy Death from hemorrhage	16	10

* Seen during a residency in obstetrics and gynecology in different hospitals, 1949 to 1952

ossible to begin the cesarean section. The interval need not be wasted; this ime is usually adequate to observe labor, to rupture membranes, and to make ndicated preparations, including cross-matching of several units of blood. Patients already in active labor or easily induced into labor by rupture of nembranes are likely to approach delivery within the two hours. Those with ervical dystocia will not deliver within two hours, or within many hours. It s particularly those nulliparas and premature multiparas with cervical dystocia vho eventually may develop tragic complications, for the mother as well as for he baby (TABLE 5), unless early delivery is accomplished.

To terminate the pregnancy is to terminate the hazard of further progression f autoextraction and fibrination, and thus to prevent development of the omplications discussed above. To delay is to risk being forced to carry out he same or more desperate surgical intervention subsequently under progressively deteriorating conditions (TABLE 5). All too often, in the case of patients vhom we are called to see, delay has already occurred. Thus we are gaining a ontinuing positive experience prompted by earlier, unhappy observations from watchful waiting" (TABLE 5) and are acquiring a pragmatic respect for early elivery.

Despite our own satisfactory experience, it is well to keep in mind that esarean section itself, in some unknown way, is not without hazard for the nitiation of defibrination.[42] Meanwhile, it is also well to note how satisactorily conservative management can progress in careful hands.[4, 18, 19]

It goes without saying that the remarkable progress made during the pas twenty years by both conservative and active approaches in maternal car owes much of its success to modern adjuncts of diagnosis and treatment and t the full use of advances in medicine, surgery, anesthesia, antibiotics, and th blood bank.

Attention is invited to the hazard of excessive transfusion. Especially i view of the danger of acute cor pulmonale, blood replacement should not exceed the blood loss.[74, 81] In times gone by, at autopsy as much as six or mor 500 ml. bottles of transfused blood could be accounted for in the engorged an distended liver.[37] Neither must the hazard of mismatched transfusion b overlooked.

The danger of excessive transfusion would seem to be even greater in amniotic embolism, with its strong tendency for splanchnic pooling of the blood an for persistence of the offending pulmonary vascular occlusions.

Hypercoagulable Fibrinogen

Drawn venous blood from women with abruptio placentae or with sever pre-eclampsia, or from dogs soon after injection of thromboplastin is likely t be hypercoagulable inasmuch as, within the drawn blood or its plasma, smal clots may form during storage in the refrigerator or when the plasma is froze and then thawed.[80, 82] Only a portion of the fibrinogen is thus removed. A cold-precipitable fibrinogen from such blood has been referred to as cryofibrinogen.[21] Whether this altered cryofibrinogen is peculiarly susceptible to fibrinolysin is a question that might be of interest to investigators of fibrinolysi in late pregnancy; it is particularly in extreme defibrination,[55] that is, especially in conditions appropriate for cryofibrinogen, that there does seem to be evidence of fibrinogenolysis.[55]

In any event, demonstration of cryofibrinogen may be indicative of an *in viv* alteration of coagulability, and this may be one aspect of the "positive phase" blood of Mellanby—an early and transient phase of hypercoagulability that follows intravascular coagulation. In severe fibrination, Mellanby's positive phase is followed at once by a "negative phase" in which coagulability is depressed.

Thus, perhaps during the early stages of fibrination of abruptio, the mother may have a somewhat increased sensitivity to a second bout of autoextraction. If so, perhaps it should be investigated experimentally whether, early in fibrination, more than one factor may conspire to make therapeutic infusion of fibrinogen hazardous (compare with FIGURE 20). Therapeutic restoration of fibrinogen overcomes refractoriness of an individual to tissue thromboplastin, and if this is given at a time when further autoextraction may still recur the result may be to render the patient once again susceptible to the hazards of fibrination.[57] In addition, there is always the possibility that, tending in the same direction, some preparations of fibrinogen may be rendered hypercoagulable during their process of preparation. Ratnoff noted that purified fibrinogen did not survive as long in a patient as fibrinogen from bank blood has been reported to survive.[59]

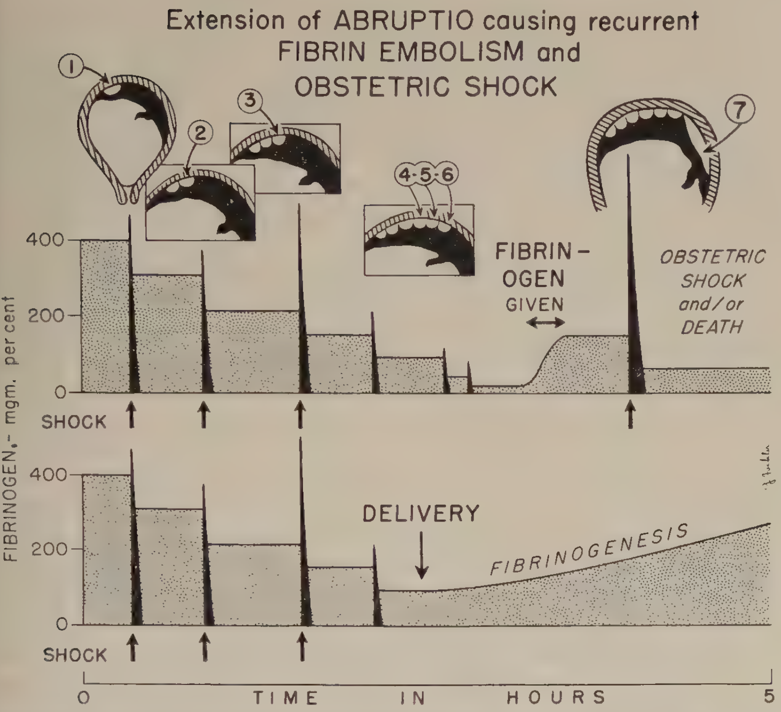

FIGURE 20. Concept of one way in which therapeutic restoration of blood fibrinogen may e hazardous to a mother with abruptio placentae if the fibrinogen is given too early, that is, f given before completion of the separation. From observation it seems probable that auto- xtraction of decidual tissue thromboplastin may recur in successive episodes as often as here may be extention of the retroplacental separation of the abruptio. Each time there nay be some variable degree of autoextraction of tissue thromboplastin into the maternal irculation. The cumulative effect upon the mother may approach afibrinogenemia. She vould then be refractory to further autoextraction of tissue thromboplastin. However if, nder these conditions, circulating fibrinogen were restored by infusion of Cohn Fraction I r of purified fibrinogen, the mother would have been rendered once more susceptible to fur- her autoextraction of tissue thromboplastin. Conceivably, if there were a marked extension f the abruptio or a peculiar episode of extension resulting in a renewed bout of marked auto- xtraction of tissue thromboplastin, then it is possible that the mother might once again ndergo severe fibrination. Conceivably any degree of complication up to obstetric shock f abruptio placentae and death could occur, as indicated in the diagram.

It should be mentioned that, although it is possible to demonstrate in animal experiments hat the individual again can become sensitive to tissue thromboplastin under the circum- tances outlined above, fortunately the sensitivity is not as great, and a much larger dose of issue thromboplastin would be required for a fatal result. The danger, therefore, is partially elf-compensating.

From Schneider,[85] *Physiologie und Pathologie der Blutgerinnung in der Gestationsperiode.* riedrich-Karl Schattauer. Stuttgart, Germany.

Hazards of Administration of Fibrinogen

Aside from the hazard of transmitting the hepatitis virus with possible de- ayed consequences or the immediate hazard of a citrate reaction during the oo-rapid administration of fibrinogen, as just described a further danger xists: that of renewed fibrination due to renewing fibrinogen.

Fulton and Page[20] re-established the sensitivity of defibrinated mice to inje tion of tissue thromboplastin by restoring fibrinogen therapeutically. Brade and I have carried out similar experiments in dogs.[87] Once defibrinated, th animals were of course refractory to thromboplastin, no matter how large th dose. When fibrinogen was restored in one of several ways, the animals agai became sensitive to tissue thromboplastin. It should be added that muc larger doses were required: for example, for a lethal effect, so that althoug sensitivity to fibrination was qualitatively restored, there was nevertheless quantitative difference.

Robert I. McClaughry, in a personal communication, describes the result o adding purified fibrinogen to citrated venous blood collected at various time after abruptio placentae, or collected from dogs after fibrination induced b tissue thromboplastin. The citrated blood or its plasma caused the adde fibrinogen to coagulate. McClaughry concluded that there must have bee active thrombin in the circulating blood after fibrination defibrination. A sufficiently low concentrations, coagulation is a function of the fibrinoge concentration itself.

Significantly, it is sometimes observed after therapeutic administration o fibrinogen that a defibrinated patient, instead of seeming to improve, ma seem to deteriorate progressively. Quite aside from the possibility of citrat intoxication from the fibrinogen preparation, McClaughry points out that administration of the substrate fibrinogen itself may have resulted in furthe fibrination. For this reason, he raises the question whether fibrinogen administered therapeutically should be administered slowly if, indeed, it seems wis to administer it at all, during the interval in which there may still be fre thrombin in the circulating blood.

The hazard of similar therapeutic restoration of fibrinogen to a woman wit abruptio, when the abruptio may still progress, is indicated diagrammaticall in FIGURE 20. Although we have called attention to this danger,[83, 85] we hav yet to become aware of an actual disaster in an obstetric patient. This i perhaps in part because, once there is appreciable defibrination, there are als other depletions of coagulation (TABLE 2), so that the individual's sensitivity although restored, may be restored only partially, and an unusually large tota dose of tissue thromboplastin would be required for lethal fibrination. Th hazard from therapeutic restoration accordingly may be more theoretical tha real.

However, a death has been reported[23] in a surgical patient with widesprea intravascular fibrin deposition that followed correction of fibrinopenia by massive restoration of fibrinogen. Apparently, then, the hazard for the patien from restoration of fibrinogen before removal of the mechanism that ca activate fibrination, sometimes can be quite real.

Correspondingly, if the administration of fibrinogen seems to be required i abruptio, we have long suggested giving this coincidentally with emptying o the uterus, not before.[74, 81, 88]

Trophoblast Embolism and Decidual Embolism

Trophoblast embolism is well known and Dieckmann suggested this as indicative of a pathway by which "tissue fibrinogen" (tissue thromboplastin) could be released into the circulation in minute amounts perhaps to cause toxemia of pregnancy. Recently, sufficiently massive trophoblast embolism to cause maternal death has been suggested in pregnancy[104] and in molar pregnancy.[46] Fibrination may have played a role.

Similar possibilities of some degree of fibrination are suggested by the finding by Park[54] of pulmonary emboli of decidual tissue in a maternal death.

The pathogenesis of the rabbit maternal death (FIGURE 8) following placental trauma is perhaps more akin to the decidual embolism than to the trophoblast embolism inasmuch as, in the rabbit, the autoextraction and the embolic material most likely came from the so-called maternal placenta.

Dead Fetus Syndrome and Fibrinopenia

Weiner *et al.*[107] discovered fibrinopenia in relation to a dead fetus carried in utero for several weeks. In this way, a fourth degree of erythroblastosis fetalis can affect not only the fetus but sometimes, secondarily, the mother as well. In this most severe degree of erythroblastosis, whether due to the Rh factor or to some other blood-grouping antigen, the macerated fetus is delivered subsequently with no gross stigmata of erythroblastosis. It is not necessary, of course, in this kind of missed abortion, for the fetus to have died from erythroblastosis fetalis in order to cause the dead fetus syndrome.

It was in a mother with such a missed abortion that Levine and Stetson[43] discovered the Rh antibody. While investigations of the Rh factor have since revealed that development of the maternal antibodies lethal to the fetus may have been induced long before the pregnancy under consideration, we can and still do wonder, with Levine and Stetson, as to how many and what kind of active materials or "toxins"[27, 28, 43, 79] may have been released into the mother from the degenerated and macerating conceptus. Thus, when allowance is made for the diverse kinds of materials that could be released in diverse ways into the maternal circulation from (1) the intervillous spaces[81] of a degenerating placenta, (2) a delayed abruptio placentae,[79] or (3) the macerated fetus and the amniotic fluid,[27, 28] it is perhaps not surprising that varying degrees of disturbance of coagulation, including fibrinopenia and thrombocytopenia, have been encountered.[27, 28, 62, 79] Release of tissue thromboplastin may account for rapid defibrination; perhaps gradual release of this or of other substances could account for gradual development of the fibrinopenia also reported.[27, 28, 61, 107]

Obstetric intervention, whether for induction of labor or for abdominal delivery, is still hazardous in missed abortion.[79] Fortunately, in the human, the tendency is for spontaneous delivery of the fetus dead in utero; therefore, development of a serious hemorrhagic disorder is not frequent. Individualization of treatment is somewhat a matter of taste, but must take into accoun cervical dystocia.[79]

The sensitized mother may develop a hemorrhagic diathesis as a result c another kind of accident; if she is transfused without due regard to her R sensitization, she may develop a transfusion reaction and, from this, fibrinopenia.

Transfusion Reactions and Fibrinopenia

Mismatched transfusions. Occasionally defibrination is caused by a transfusion reaction;[39] we have observed nearly complete defibrination followin transfusion of a portion of a unit of blood.[83] Fibrinopenia from a transfusio reaction on the medical ward will usually have no hemorrhagic sequelae. I our patient bleeding did develop from a chemical ulceration in the vagina an from the vena-puncture point. Unless tests are carried out, disturbances o coagulation may well go as an undetected result of the transfusion reaction yet the same reaction occurring during surgery or in relation to obstetric delivery may permit tragic bleeding from lacerated or from incised tissue surface

The mechanism of the defibrinogenation is not clear. Conceivably fibrination defibrination could result from some peculiarly active antigenic or othe mechanism for lysis of platelets. A thromboplastic activity of the lysed ghost or erythrocytes or of the leukocytic cellular debris has been suggested.[94] Neither in human[5, 28, 39] nor in animal experimental transfusion reactions[26, 28] ha fibrinolysis been observed.

Hemolysis is, of course, a far more severe component in a transfusion reaction than in fibrination of abruptio placentae[74] or in eclampsia.[76] In a transfusion reaction, disseminated pulmonary vascular occlusions from antigenically agglutinated erythrocytes, or of their ghosts, is probable; perhaps it is pulmonary hypertension due to this kind of disseminated pulmonary embolism which in our own experience with rapid transfusions of human blood into dogs, cause acute cor pulmonale.[80, 81] However, there was no major disturbance of coagulation, which may occur with slower transfusion.[28]

Autotransfusion from fetus to mother. "The fetus can bleed."[5] Although n case of fibrinopenia is yet reported as a result of autotransfusions from the fetus into the mother, such will undoubtedly occur. Well-authenticated observations of accidental transfusion by transplacental escape of fetal blood int the mother with resultant sensitization are already known.[5]

Pitocin

We have developed an innate revulsion to the use of Pitocin to stimulate labor in abruptio placentae or in the dead fetus syndrome. Perhaps we are unduly sensitive because of the loss of a patient[79] (TABLE 1). Actually that patient was lost, not because of the Pitocin, but because of poor obstetric management; any one of her several doctors alone would have obtained better results. She had reverted to laborlike contractions before the Pitocin and had active labor for four hours after the Pitocin stimulation was discontinued, but without further dilatation of the effaced and three-cm. dilated cervix—there was cervical dystocia of premature labor in a multipara. The labor was adequate to rupture the uterus. With Page, we may state that the rupture was not the result of using Pitocin, which had been discontinued long since. Indeed, it may be wondered, with the high uterine tonus of the uterus of abruptio, how effective Pitocin would be for establishment of labor in any event. Mere thought of the potential hazard of labor causing rupture through an area of Couvelaire-type infarction is enough to give pause. The concept of possible further autoextraction by one mechanism or another, with prolonged or unproductive labor, does not arouse confidence in the use of pitocin or other stimulation in abruptio placentae or in the dead fetus syndrome.

Summary

Fibrination defibrination can and sometimes does occur in abruptio placentae and in certain other acute accidents of late pregnancy.

Fibrination is coagulation within, and of, the circulating blood. The fibrin is deposited out of the blood. To the degree to which there is fibrination, there must be, at the same time, defibrination or fibrinopenia, that is, defibrinogenation. The blood remains liquid, continues to circulate, and may fail to support hemostasis.

Measurement of the defibrination provides a convenient index for a panhemostatic depletion, including an acutely acquired thrombocytopenia.

Not of least interest is an acute cor pulmonale that can result from disseminated pulmonary vascular obstruction by characteristic fibrin occlusions.

Perhaps because acute cor pulmonale and obstetric shock of abruptio placentae are caused by fibrination, these disorders may be much more ephemeral than is the acute cor pulmonale and the obstetric shock of amniotic fluid embolism. Amniotic embolism provides an excellent control without necessarily involving blood coagulation; indeed, coagulation is sometimes inhibited. The occluding pulmonary intravascular debris of amniotic embolism is relatively persistent.

In fibrination, the systemic pressure may be supported by a compensating vasopressor agent, serotonin, released from platelets during fibrination. In addition, the fibrin occlusions of abruptio are soon lysed away.

Patients with abruptio can usually be expected, under adequate treatment, to survive the acute episode. Maternal and fetal mortality and morbidity are largely the result of secondary complications. These are reduced to a minimum by obstetric management for prompt delivery.

One complication is hemorrhage. Exsanguination and shock from this or from other, simple hemorrhage can also lead, in some other way, to another mechanism of fibrination defibrination. Fibrinolysin may be activated and, in the blood, this acts primarily on fibrin, not on fibrinogen. Nevertheless, there may be increased hazard of further hemorrhage because of fibrinolysis of hemostatic thrombi.

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ETIOLOGY OF AFIBRINOGENEMIA: FIBRINOGENOLYTIC AND FIBRINOLYTIC PHENOMENA*

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The viewpoint that intravascular coagulation is a starting point in the etiology of afibrinogenemia is widely held by many investigators in the field. However, it seems insufficient as an explanation for the extent of fibrinogen loss, as well as for some of the other results obtained in this laboratory. Moreover, data presented in the monograph *Proteolytic Enzymes* published by The New York Academy of Sciences in 1957 would seem to argue against the method of intravascular clotting as the complete explanation for the etiology of afibrinogenemia. While investigators represented in that publication were not concerned with the mechanism involved in this process, their data may have some bearing on the subject. Bramble,[1] in experimental work on dogs, showed that if 100 units of thrombin were injected intravenously over a period of 1 hour, no decrease in fibrinogen was observed and the animal experienced no harmful effects. When the animals subsequently were sacrificed, no fibrin deposits could be found in the tissues. However, if the 100 units of thrombin were administered in 5 min., the entire vascular tree was clotted and the animal died instantly. In view of these results it is difficult to conceive of a mechanism by which tissue thromboplastin could produce thrombin intravascularly at such rate that all the fibrinogen would be removed by coagulation and yet no occlusion of blood vessels would occur.

On the other hand, Ambrus *et al.*[2] demonstrated in both animals and humans that intravenous administration of fibrinolysin produced a decrease in fibrinogen and inhibitor levels and frequently was accompanied by a drop in blood pressure. These are the same findings that occur in obstetrical afibrinogenemia. Therefore it is suggested that, while partial intravascular coagulation may occur in a number of obstetrical accidents, most of the drop in fibrinogen level is due to the hydrolysis of fibrinogen by fibrinolysin and/or other proteolytic enzymes.

Profibrinolysin, which occurs normally in blood plasma, has been shown to be activated *in vivo* by a number of processes, many of which could be present even during normal labor and delivery. These include such factors as anxiety, severe exercise, and administration of adrenaline. However, activation by tissue extracts is of particular interest in these cases of obstetrical accident.

A possible mechanism for the activation of the enzyme and for the subsequent hydrolysis of fibrinogen and fibrin by the fibrinolysin so produced, is shown in FIGURE 1.

TABLE 1 shows results obtained for fibrinogenolytic and fibrinolytic activity of tissue extracts alone and of a partially purified profibrinolysin activated by

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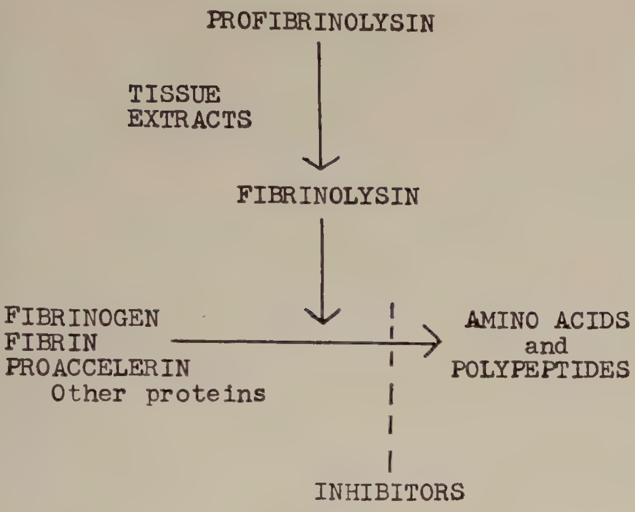

FIGURE 1

TABLE 1
ACTIVITIES OF TISSUE EXTRACTS

Tissue	Fibrinogenolytic units		Fibrinolytic units	
	Active enzyme	Activator	Active enzyme	Activator
Myometrium				
M. B.	1.5	1.8	0.10	0.85
M. C.	1.3	0.0	0.08	0.02
M. D.	0.9	0.4	0.10	0.32
R. G.	1.2	0.0	0.30	1.17
J. V.	1.2	0.4	0.08	0.00
M. H.	0.8	0.7	0.42	1.12
Average	1.15	0.55	0.18	0.58
Decidua				
M. B.	1.1	2.0	0.08	0.02
M. C.	1.6	1.2	0.10	0.00
M. D.	2.3	1.1	—	—
R. G.	1.0	0.0	0.08	0.02
J. V.	1.1	0.0	0.10	0.00
M. H.	1.2	1.1	0.08	0.02
Average	1.4	0.9	0.08	0.01
Placenta				
M. B.	2.0	0.6	0.08	0.10
M. C.	2.5	2.3	0.18	0.00
M. D.	2.3	0.0	0.08	0.10
R. G.	1.1	0.5	0.08	0.02
M. H.	2.6	0.0	0.08	0.00
16 weeks	2.4	3.2	0.10	0.08
Average	2.15	1.05	0.10	0.05

these tissue extracts.[3] The units of fibrinogenolytic activity and fibrinolytic activity are not expressed in the same terms and, therefore, the figures themselves are not comparable. However, it is obvious that significant fibrinogenolytic activity was found more frequently in the tissues themselves and that this activity was produced from the profibrinolysin by almost all of the samples of tissue extracts examined. The myometrium itself was the only one of the tissue extracts that showed any significant fibrinolysis and the only one that produced this activity from the proenzyme. The greatest fibrinogenolytic activity and activation was produced by placental extracts, the decidual extracts coming next in order. These are the substances that Schneider[4] postulates may be forced into the maternal blood stream in cases such as abruptio placentae. Therefore, along with thromboplastin, both a proteolytic enzymes and an activator of the fibrinolytic system are introduced into the maternal circulation. The entrance of these tissues into the blood stream may then initiate hydrolysis of the fibrinogen, which results in afibrinogenemia.

While the greatest number of patients with low fibrinogen levels are found to have such conditions as abruptio placentae and long-standing fetal death, they are by no means limited to these categories. TABLE 2 shows the cases that have been studied at the Columbia-Presbyterian Medical Center during the last 4 years and are known to have had fibrinogen levels below 200 mg. per cent. Since not all of these required the administration of fibrinogen to control bleeding, the second column shows the number of those that did. No deaths from hemorrhage occurred in these 42 patients, although the 2 patients with leukemia and the one diagnosed as thrombotic thrombocytopenic purpura died from other manifestations of their disease within a few weeks or months of delivery.

One of the arguments used most effectively against the theory of a destruction of fibrinogen by the fibrinolytic enzyme has been the failure of so many investigators to find any evidence of an active fibrinolysis. Experience in this laboratory in studying serial blood samples on those patients who develop hypofibrinogenemia would make it seem a somewhat fortunate coincidence that so many investigators have been able to detect it. Frequently, a sample of

TABLE 2
OBSTETRICAL HYPOFIBRINOGENEMIA

	Number of cases with fibrinogen less than 200 mg. %	Number of cases requiring fibrinogen to control hemorrhage
Abruptio placentae	17	8
Long-standing fetal death	8	2
Retained secundines	5	3
Normal spontaneous delivery	4	3
Placenta praevia	3	1
Elective 2° section	2	2
Leukemia	2	1
Thrombotic thrombocytopenic purpura	1	0
Total	42	20

TABLE 3
NORMAL SPONTANEOUS DELIVERY

Time	Fibrinogen (mg. %)	Plasma clot lysis
June 21 5:30 A.M.	190	++++++ (5 hours)
8:30 A.M.	122	+++ (24 hours)
11:00 A.M.	117	++ (24 hours)
4:00 P.M.	131	+ (24 hours)
June 22	323	− (24 hours)

blood drawn on a patient at one time will show strong fibrinolysis while, a short time later, a sample with a lower fibrinogen level will fail to demonstrate it. Fibrinolysin is an extremely labile enzyme and disappears rapidly from the circulation. Fairly frequent detection has been possible at Presbyterian Hospital, New York, N. Y., only because it has become an established practice there to draw blood samples for fibrinogen studies on all patients who are suspected of having abruptio placentae, who have a dead fetus *in utero*, or who have any unusual display of bleeding.

TABLE 3 shows the fibrinogen levels and plasma clot lysis obtained on serial samples from a patient who delivered normally, but bled considerably after delivery. The fibrinogen level at this time was 190 mg. per cent, but within 5 hours serially diluted plasma clots all showed complete lysis. Subsequent samples showed decreasing lysis, although fibrinogen levels fell considerably lower. From this and other patients it would appear that lysis is most easily detectable before fibrinogen levels reach their lowest figures.

Because of the transient nature of this enzyme, it seemed essential to find means other than observation of clot lysis for investigating the problem. No single determination has proved adequate, but a combination of several assays seems to give a fairly clear picture of what is taking place in the blood stream. The following methods for studying the plasma have been used: (1) fibrinogen—the method of Ratnoff and Menzie;[5] (2) clot lysis—the observation of serially diluted plasma clots in modified Coon's method;[6] (3) free profibrinolysin—hydrolysis of casein by plasma activated with streptokinase;[7] (4) total profibrinolysin—hydrolysis of casein by an isoelectric precipitate (from plasma) activated with streptokinase;[7] and (5) inhibitors—the difference between the *total* and *free* profibrinolysin.[7]

Since the free profibrinolysin—that found in the plasma itself—depends directly on the level of profibrinolysin but inversely on the inhibitor level, it has little meaning alone. When the inhibitors are removed, however, by precipitating the enzyme isoelectrically, and the activity is determined in the redissolved precipitate, a truer estimate of the profibrinolysin can be made. The difference between these two levels is a measure of the inhibitors that were discarded in the supernatant fluid along with the albumin fraction of the plasma. These determinations have been made on serial blood samples from most of the forty-two patients with low fibrinogen levels, as well as from many normal subjects, who have been studied in order to establish normal ranges.

FIGURE 2 shows the results obtained on two cases of abruptio placentae.[7]

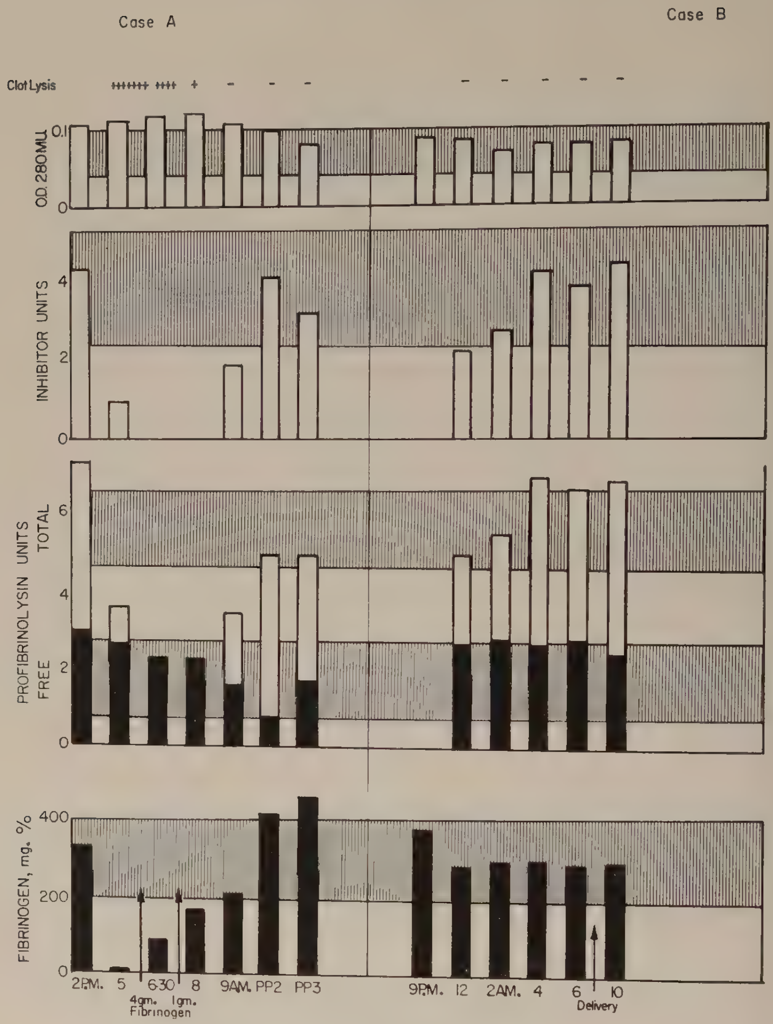

FIGURE 2. Abruptio placentae.

Case A developed afibrinogenemia, while the fibrinogen levels of Case B remained well within normal limits. The first sample of Case A was drawn at the time of cesarean section because an abruptio placentae was suspected. A live healthy baby was delivered, and closure was accomplished without any great difficulty, although some unusual oozing was observed. Three hours later the patient's blood pressure suddenly dropped and fluid blood started to gush from the vagina in the manner typical of these cases of afibrinogenemia. Fibrinogen was administered promptly when it became evident that the level had dropped precipitously. After the fourth gram the fibrinogen level had risen somewhat, and a complete reversal of the patient's condition was obtained after the fifth gram. The patient's blood pressure rose, clots began to appear in the blood, and bleeding gradually subsided. From the figure it is obvious that between cesarean section and the beginning of hemorrhage not only the fibrinogen level, but also the total profibrinolysin and inhibitor levels, fell. This is interpreted as evidence that the precursor was activated during this period and that the active enzyme hydrolyzed the fibrinogen in the blood stream. The fall in inhibitor level may be due to the activation process, since it has been suggested that tissue extracts activate the enzyme system by adsorption of the inhibitors on the microsomes and mitochondria of these extracts.[8] It may be due also to hydrolysis by the active enzyme, or to a combination of these processes.

This is a typical picture obtained with these cases of afibrinogenemia—a drop in fibrinogen is accompanied by a drop in total profibrinolysin and inhibitors. However, as seen in Case B, where the profibrinolysin and inhibitor levels remained within normal limits and even rose somewhat before delivery, the fibrinogen level also remained extremely stable after a slight initial drop. Unfortunately, insufficient blood was obtained on the first sample to make complete studies of the fibrinolytic system. It may be that a slight drop in all factors occurred between the first two samples.

Case A presents other interesting points in the problem of obstetrical afibrinogenemia. Here, obviously, emptying the uterus did not prevent the development of a bleeding tendency. The fibrinogen level was perfectly normal at the time of cesarean section, but the syndrome developed 3 hours later. Apparently some process had been initiated before delivery that was not halted by the removal of the fetus and placenta. This time factor in itself is interesting, since it is more indicative of an enzymatic hydrolysis process than of a coagulation process. We were fortunate to have obtained an early level on this patient. So often blood is drawn only after hemorrhage has begun and the fibrinogen level is already low. However, by figuring back on many of the cases of abruptio placentae, it was found that the lowest level of fibrinogen usually occurred 5 to 7 hours after the first symptoms of abruptio placentae were noted.

Three cases in which fibrinogen levels were low but hemorrhage was controlled by means other than fibrinogen administration are presented in FIGURE 3.

The fetus of the first patient had been dead *in utero* for approximately 4 weeks when a blood sample was first studied. All factors were within normal limits at this time. A week later the fibrinogen level had fallen to 154 mg. per cent, and the total profibrinolysin and inhibitor levels were considerably below normal limits. The fibrinogen level continued to fall until delivery and for some hours afterwards, while profibrinolysin and inhibitors remained low. Bleeding, which occurred about 3 hours postpartum, was controlled with fresh whole blood. The recent paper of Lewis *et al.*[9] lends efficacy to the use of fresh blood in these cases, since these authors have shown that proaccelerin is

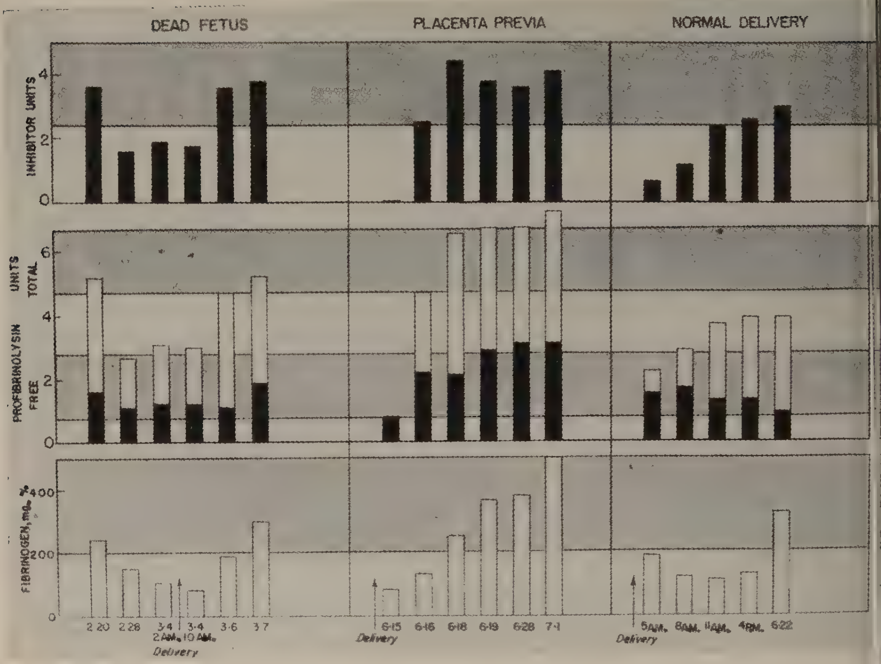

FIGURE 3

also destroyed by fibrinolysin. Since proaccelerin is obtained only in fresh blood, its administration may help to control bleeding where fibrinogen is somewhat low, but not severely depleted. By the second postpartum day all factors of the fibrinolytic system were essentially normal, and the fibrinogen and profibrinolysin continued to rise.

The second case on this figure shows the results obtained from a patient with a diagnosis of placenta praevia. Hemorrhage became such a problem at the time of a tertiary cesarean section, when the first blood sample was drawn, that a hysterectomy was performed. Fibrinogen, total profibrinolysin, and inhibitor levels were low, but rose gradually until high levels of all factors were reached.

In the third section are presented the results in the normal spontaneous delivery discussed earlier in reference to the lability of the enzyme. Here, also, low fibrinogen levels are accompanied by low total profibrinolysin and inhibitor levels.

FIGURE 4 shows results on a patient who presented herself at Emergency Admitting with a chief complaint of bleeding from a tooth socket following extraction 3 days previously. Her body had numerous areas of ecchymosis. She gave a history of an abortion 2 months previous to admission, and her uterus was found to be enlarged to an 8 weeks' size. An A-Z test was reported positive. The fibrinogen level was 50 mg. per cent, and all other factors of the

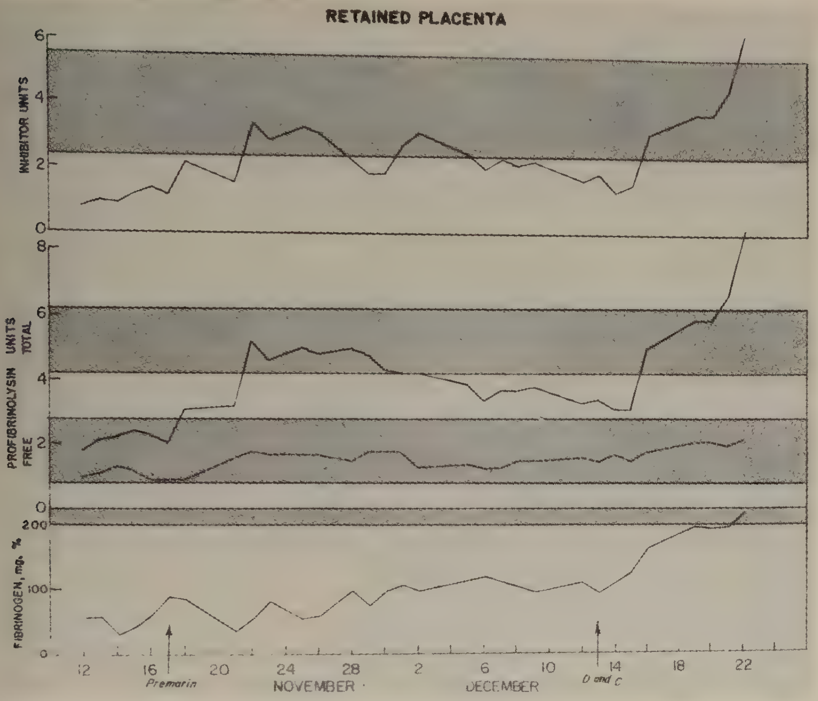

FIGURE 4

fibrinolytic system were found to be low. Thereafter, levels were followed almost daily for the 6 weeks she remained on the ward. On the fifth day after admission, Premarin was administered, with the cessation of oozing from the tooth socket. This was accompanied by a slight rise in total profibrinolysin and inhibitors, which persisted for about a week. The fibrinogen levels showed considerable fluctuation, but a gradual climb resulted in levels of about 100 mg. per cent by the time consistently negative results were obtained on A-Z tests, and it was decided to perform a dilatation and curettage. At operation, large amounts of placental tissue were removed; on microscopic examination, this tissue showed hyalinized chorionic villi, viable and nonviable decidua, and trophoblasts, some of which were active in appearance while others were inactive. Apparently the fetus had been expelled at the abortion 2 months prior to the patient's admission, but the placenta had remained *in situ*. No undue bleeding was encountered during the operative procedure. While 2 or 3 days were required in this case for the fibrinolytic system to begin to rise, within a week all factors had returned to normal and continued to increase to somewhat elevated levels.

It is interesting that the length of time required for factors to return to normal seems to be related directly to the length of time that a state of hypofibrinogenemia has endured. In cases such as abruptio placentae, where low levels have persisted for only a few hours, the return is rapid. Usually less than 12 hours suffices to produce normal figures. In cases of long-standing fetal death, where fibrinogen may have been low for 1 to 3 weeks, 2 or 3 days are usually required before the fibrinolytic system returns to normal. In the last case discussed, where the condition had persisted for a period of 2 to months, normal values were not re-established for a week after removal of the placental tissue.

Only a few representative cases of the many studied have been presented in this discussion. All have shown approximately the same pattern. Where fibrinogen levels have been severely depressed, the total profibrinolysin level has been low, as have been the inhibitor levels. Where the enzyme and inhibitor levels have remained high, fibrinogen levels also have remained normal in spite of occasional severe bleeding from other causes. This same pattern also has been encountered in cases of surgical hemorrhage from hypofibrinogenemia.

It is felt, therefore, that while intravascular coagulation may take place in the initial phases of obstetrical accidents, the most probable mechanism for the depletion of fibrinogen is its removal by hydrolysis by a fibrinogenolytic enzyme. The occasional presence of an active fibrinolysin in these cases supports this theory, but the consistently low profibrinolysin and inhibitor levels are even more indicative that a fibrinolytic process is involved. This decrease in profibrinolysin suggests that the enzyme has been activated previously. It is then utilized to destroy fibrinogen and is rapidly denatured. The denaturation would prevent the observation of an active lytic process except for a short interval of time. Since changes in fibrinogen, profibrinolysin, and inhibitor levels occur concomitantly, it seems probable that a fibrinogenolytic process is responsible for the destruction of fibrinogen and the other clotting factors, as has been observed in these cases of afibrinogenemia from obstetrical accidents.

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CLINICAL CONSIDERATIONS IN HYPOFIBRINOGENEMIA

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For many years clinicians have remarked that in intrapartum and postpartum hemorrhage the shed blood occasionally fails to clot. On the basis of present evidence, the clotting defect appears to be due chiefly to a reduction in the plasma fibrinogen concentration. Although other factors of blood clotting may be decreased, especially platelets, AC globulin, and prothrombin activity, the reduction in these components never reaches a hemorrhagic level. In the majority of instances the fibrinogen value is somewhat less than 100 mg./100 ml., although in the rare case no fibrinogen may be detected in the blood. A fibrinogen concentration below 150 mg./100 ml. in the pregnant patient now is generally regarded as abnormal.[1] It has been demonstrated that hypofibrinogenemia, a more acceptable term than afibrinogenemia, may develop in the more severe form of abruptio placentae, long-standing fetal death *in utero*, and amniotic fluid embolism.[1-4]

Etiology

How hypofibrinogenemia develops remains in the realm of theory. The decrease in fibrinogen levels is believed to be due to excessive utilization rather than to a failure in the production of this plasma protein. According to the more commonly held view, the circulating fibrinogen presumably is consumed through the medium of intravascular clotting initiated by the introduction of a thromboplastinlike substance from the uterus into the maternal circulation.[5, 6] Fibrinolysin (plasmin) or a related enzyme must be given consideration in the etiology of the abnormal clotting. Certainly, fibrinolytic activity is detected frequently and undoubtedly enhances the hemorrhagic diathesis. In patients with hypofibrinogenemia seen in this clinic, the lytic factor has been found to be active against both fibrinogen and fibrin. Whether this substance is liberated directly from the uterus, whether its precursor, plasminogen, is activated by a factor released from the uterus, or whether it is an effect of intravascular coagulation remain open questions. It is interesting to note that plasminogen activated by streptokinase will markedly depress the fibrinogen concentration *in vivo.* Nevertheless, a patient is seen occasionally who continues to ooze vaginally for 2 to 4 hours after delivery, despite the fact that her plasma fibrinogen concentration has been restored to normal and, in fact, sometimes to extremely high levels (800 to 900 mg./100 ml.). Blood samples taken during this period of concern and assayed in this laboratory have shown a high degree of fibrinolytic activity, offering further evidence that the latter may be important in the etiology of the coagulation defect. Whatever may be the cause of hypofibrinogenemia, the role played by fibrinolytic activity with respect to the integrity of clot formation at the placental site cannot be dismissed.

Incidence

The incidence of cases of hypofibrinogenemia in a clinic will depend to a degree on the number of Rh-negative, hypertensive, and toxemic patients, for they will influence the incidence of fetal death *in utero* and abruptio placentae. Surveys have shown that some degree of hypofibrinogenemia will occur in 3 to 5 instances per thousand deliveries.

Clinical Conditions Associated with Hypofibrinogenemia

Premature separation of the placenta, which occurs in 1 to 2 per cent of all pregnancies, accounts for the majority of cases that develop a coagulation defect. This defect has never been seen in the milder forms of abruptio placentae, and is restricted to patients who present a rather characteristic picture that is, patients who, in the latter weeks of pregnancy, are seized with moderate to severe abdominal pain, usually accompanied by vaginal bleeding, and who enter the hospital in some degree of shock. Physical examination reveals a spastic, tender uterus, indicative of extensive concealed hemorrhage. Fetal heart sounds frequently are absent or weak and irregular. If the uterus could be observed, it would present a mottled appearance due to the formation of hematomas beneath the serosa (Couvelaire uterus). There is often little or no urine in the patient's bladder, and what is present usually contains albumin, casts, and red cells. The degree of shock seems out of proportion to the estimated amount of external bleeding. In this clinic, hypofibrinogenemia has been detected in one third to one half of the patients with this clinical picture. In the main, the mortality from premature separation of the placenta resides in this severe group, which comprises 10 per cent of the patients with abruptio placentae.

A coagulation defect associated with fetal death *in utero* often is heralded by the appearance of ecchymoses and bleeding from the mucous membranes. It is perhaps well to emphasize that 75 to 80 per cent of these patients will not develop a clotting deficiency, principally because, in most instances, labor ensues within a month after the fetus dies. For some unexplained reason, the condition is rarely encountered prior to the twentieth week of pregnancy, and only in cases where the dead fetus has remained *in utero* for at least 5 to 6 weeks. Evidence has been presented that indicates that a clotting defect can develop in 40 per cent of the cases that retain a dead fetus beyond 6 weeks.[8] Frequently these cases are associated with Rh incompatibility, primarily because the latter commonly results in fetal death *in utero* between the fifth and eighth months, when spontaneous onset of labor is least likely. Hypofibrinogenemia may develop before the onset of labor, or its appearance may be delayed until the intrapartum period.

Amniotic fluid embolism, although a rare complication, is being reported with greater frequency. It is now accepted that the sudden appearance of shock and severe respiratory distress during an otherwise normal labor is pathognomonic of this syndrome. A factor that seems to be of etiological importance in the development of this condition is a rapid and tumultuous labor, which may be precipitated by the overenthusiastic use of pituitary preparations. In patients surviving the initial embolic insult, hemorrhagic manifestations may develop. In addition to uterine hemorrhage, there may be generalized bleeding from mucous membranes, indicating a clotting defect.

Diagnosis

Besides the clinical picture described above, the "clot observation test" has proved to be a rapid and practical means for the recognition of the hypofibrinogenemic state.[1] This simple test involves the withdrawal of 5 ml. of the patient's blood into a clean, dry test tube and observing it at frequent intervals for clot formation and stability. Normal blood will clot within 8 to 12 min., and the clot will remain intact for at least 24 hours. In the hypofibrinogenemic cases the blood may not clot or, if it does, it may undergo partial or complete dissolution within 30 to 60 min. The latter finding is indicative of hypofibrinogenemia and the likely presence of fibrinolytic activity. This test may be conducted at the bedside; the blood taken for cross matching may serve for the test. A more detailed laboratory analysis of the fibrinogen concentration of the patient's plasma confirms the diagnosis, utilizing the estimation of tyrosine activity. Shorter methods recently have been described, yielding semiquantitative approximations of fibrinogen concentration.[9, 10]

Treatment

At the Boston Lying-In Hospital the preferred treatment of severe premature separation is the induction of labor by artificial rupture of the membranes and delivery per vaginam. Because of the extent of the bleeding, one is tempted, when the patient is first seen, to perform an immediate cesarean section, especially if the fetal heart sounds are still detectable. The initial problem in management, however, is to ascertain and correct any clotting defect and to combat shock. To attempt to deliver the patient before this has been done is to invite uncontrolled hemorrhage. Blood should be administered rapidly in amounts sufficient to restore the blood volume, and the intravenous infusion of fibrinogen (Cohn's Fraction 1) can be relied upon to raise the fibrinogen concentration to normal levels. In the average case, 1,500 to 2,000 ml. of blood and 4 to 8 gm. of fibrinogen usually are required. A rule of thumb is to give 1 to 2 gm. of fibrinogen with each transfusion. Overtransfusion of these patients has been of little concern in this clinic, for the blood shed is much greater than is often appreciated. Retroperitoneal hematomata are common, and the amount of concealed blood is often considerable. Even patients who receive 4 to 6 transfusions frequently leave the hospital several days after delivery with a hemoglobin of less than 10 gm.

According to this method of management, cesarean section is restricted to cases in which effective labor does not ensue within 6 to 8 hours after amniotomy and before intrauterine infection becomes an added hazard. With the treatment outlined and the clotting mechanism restored, operative intervention can be instituted without danger of serious bleeding. This policy has been questioned on the ground that any delay in delivering the patient will allow the clotting mechanism to deteriorate further. Those who regard immediate cesarean section as the treatment of choice submit as their rationale the fact that hysterectomy can be performed easily if uncontrollable hemorrhage persists after the uterus has been emptied. However, this reasoning fails to take cognizance of the fact that at the time of hospital admission the patient's blood is, or is on the verge of, becoming incoagulable. Furthermore, bleeding in these severe cases, which in many instances is attributed to uterine atony, in all probability is the result of a defect in the clotting mechanism. Moreover, experience has shown that the coagulability of the undelivered patient's blood can be restored to normal by the administration of blood and fibrinogen.

The question most commonly posed in the management of patients with fetal death *in utero* is whether the pregnancy should be terminated before the possible appearance of a hemorrhagic diathesis. Despite the fact that a prepartum clotting defect may develop, the preferred policy is one of watchful expectancy. The majority of patients will enter spontaneous labor and deliver within a few days or three or four weeks after the disappearance of fetal heart sounds and before the clotting defect develops. At this early stage of pregnancy, induction of labor is at best an uncertain procedure, and may lead to complications. Furthermore, experience has shown that a hemorrhagic diathesis can be controlled by the prepartum and intrapartum administration of fibrinogen.

After fetal death and until the patient is delivered, she should have a weekly clot observation test and, ideally, a determination of the plasma fibrinogen concentration. She should be cautioned to report immediately any bleeding from the reproductive or gastrointestinal tract or into the subcutaneous tissues. Spontaneous correction of the fibrinogen concentration before delivery has not been seen, and the levels may continue to fall during labor. Cesarean section is restricted to the rare patient in this group in whom excessive bleeding recurs after blood and fibrinogen therapy, and in whom induction of labor is not feasible due to cervical closure.

The treatment of amniotic fluid embolism is confined initially to the administration of oxygen and the use of conventional measures to combat shock. Because of the rapidity of the labor, the cervix is found frequently to be fully dilated; if feasible, delivery should be performed in an effort to salvage the infant. If the above supportive measures are sufficient to prolong the mother's life, a delayed complication in the form of uncontrolled postpartum hemorrhage should be anticipated. The reproductive tract should be explored gently at the time of delivery to exclude vaginal and cervical lacerations as a cause of postpartum hemorrhage, should it develop. If bleeding does ensue and a defect in the clotting mechanism is demonstrated, the patient may survive if treated energetically with transfusions and fibrinogen replacement.

Summary

Hypofibrinogenemic states have been identified in severe premature separation of the placenta, amniotic fluid embolism, and where the fetus dies *in*

utero and is retained for several weeks. The diagnosis is established by a simple bedside test, namely a "clot observation test." Failure of the blood to clot is evidence of a relative afibrinogenemia. Where the fibrinogen values are borderline (100 mg. ±), or in the presence of a circulating fibrinolysin, or both, the blood may clot and undergo fragmentation and dissolution within 60 min. after withdrawal. Clot dissolution indicates a defective hemostatic mechanism.

Blood transfusions with supplementary fibrinogen are indicated. Where bleeding is active, it is impossible to restore the fibrinogen levels with transfusion alone. The amount of Fraction 1 administered will vary, depending upon the degree of defibrination. Frequent clot observation tests will serve to indicate the amount of fibrinogen needed. Usually 4 to 10 gm. of fibrinogen will be required to establish a stable clot. With restoration of the clotting mechanism to normal, the patient may be delivered safely by the appropriate obstetric procedure.

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Discussion

MARIO STEFANINI AND RINALDO A. TURPINI (*Joseph Stanton Memorial Laboratories, St. Elizabeth's Hospital, Boston, Mass.*): We wonder with Howard C. Taylor whether, when we speak of the fibrinogenopenic syndromes of pregnancy and delivery, we are not grouping under a single definition a number of conditions that may recognize multiple causes and mechanisms. The passage into the maternal blood stream of thromboplastic material from the uterus, placenta, and dead fetus; amniotic embolism (especially after sensitization);

and hemorrhagic shock all appear prominent among the causes of fibrinogenopenia. Oscar D. Ratnoff also has pointed out the potential role of bacteremia and of the entrance into the blood stream of chemicals, as in criminal abortion. Whether the pathogenetic mechanisms involved are actually different, and whether differences observed are more apparent than real and are due to the timing of the observations during the fibrinogenopenic crisis cannot be decided without further thought and work. In any case, two mechanisms probably are significant: intravascular clotting and fibrinolysis, which seem intimately associated, although of changing importance in each instance and at various intervals of time during the crisis. Perhaps their respective roles may not be clearly separated, as the two mechanisms may follow or even precipitate each other. The general feeling seems to be that intravascular clotting may be the most important phenomenon.

Still, after Louise Phillips' quite convincing observations, one cannot dismiss lightly the role of fibrinolysis. We have found evidence for intravascular clotting and for fibrinolysis associated in the various experiments leading to fibrinogenopenia in animals, probably because the separation of thromboplastic and fibrinolytic constituents of the tissue preparations used is quite relative. A similar situation prevailed in hemorrhagic shock.

With reference to hemorrhagic shock, our studies bring out the possibility that such phenomena as fibrinogenopenia and thrombocytopenia, found in accidents of pregnancy, might be caused by bleeding rather than induce it. At the present time we cannot establish the significance of this mechanism in the average case of fibrinogenopenia, but we feel that it should be considered in some instances when anatomical conditions explain the sudden occurrence of massive uterine hemorrhage. Finally, we have found antithromboplastin anticoagulants following the infusion of tissue thromboplastin in animals. Thus, the finding of a circulating antithromboplastin in patients points to thromboplastic infusion as the pathogenetic mechanism of fibrinogenopenia.

We were impressed by Schneider's accurate analysis of the dynamic aspects of the fibrinogenopenic syndromes. Their relation to the phenomena of intravascular clotting, with consequent release of platelet constituents and activation of plasmatic factors, should represent a fruitful future field of research. Marked pulmonary hypertension in dogs receiving intravascular thromboplastin suggested to us a possible role of serotonin or of related substances, which seems to deserve thorough investigation.

Two final points should be made. We should perhaps drop the misnomer of "fibrinogenopenia" and adopt the more encompassing and more fitting definition of "hemostatic breakdown" or similar wording, until such time as we are able to obtain a better insight into the pathogenesis of each case of fibrinogenopenic accident of pregnancy and delivery. Even more important, an agreement as to the significance of the techniques employed seems vital. Their standardization will also remain of great concern until such time as more specific and adequate procedures may be developed.

Part V. Aspects of the Gravid Uterus

GESTATION MECHANISMS*

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The Background of our Knowledge

Until the time of Heape in the latter part of the Nineteenth Century, knowl dge concerning most reproductive processes was fragmentary at best. During the lifetime of but one man, F. H. A. Marshall, who was student of Heape and in turn a distinguished teacher, the subject of the physiology of reproduction became an entity. It is one that has yielded a rich body of knowledge of those mechanisms that give rise to the union in mammals of two gametes and, through a series of complicated and interrelated changes within the womb, result in living offspring.

The discovery in 1917 of the estrous cycle in the guinea pig by Stockard and Papanicolaou served to awaken interest in the United States in the physiology of reproduction. It led to the discovery of the sex and pituitary hormones and the appreciation, still incomplete, of the mechanisms of their actions. This entire area of study continues to be pursued in a number of directions. Scholars in animal husbandry, veterinary medicine, gynecology, and obstetrics have made, as many readers of these pages will be aware, enormous strides in the fields of fertility and of infertility. These have had great social, economic, and medical consequences. The relation of sex hormones and reproductive states to the differentiation and the growth of tissue holds the attention of many students. The relation of sex hormones to carcinogenesis, genetics, and the structure and function of protein complexes throughout the body, as well as to water, salt, and other forms of metabolism, likewise furnish active fields of research. Study of the mechanisms of gestation continues to occupy the active attention of students everywhere.

As in so many other fields of human endeavor, we tend to break up complex mechanisms into their simpler components. By doing so, we hope to achieve a fuller understanding of a more complicated whole. There is a tendency here, however, to become increasingly narrow. As a result, we fail to see the larger relations. It is these that I shall endeavor to present in this paper.

The uterus serves three related and interdependent functions. It provides periodically for the reception and implantation of fertilized ova; it grows and adapts to, thereby accommodating, the growing products of conception; and it assures delivery at term of offspring capable of ultimate survival in the ex- ernal world. Let us look first at the mechanisms of uterine accommodation.

Uterine Accommodation of the Products of Conception

There are three main characteristics in the manner by which a uterus adapts itself to its contents during pregnancy. These three phases we call uterine accommodation. One phase, before the time of implantation, is a period of *uterine preparation* through progestational proliferation of all elements in the uterus and of sensitization of the proliferated endometrium for nidation. At this time the uterus fails to increase noticeably in weight, although it contains more cells than before. Immediately after implantation, the uterus begins to grow in size, for its weight, minus the weight of its contents, becomes progressively greater as time goes on. This is the time of *uterine growth.* At a later time the uterine growth rate diminishes, even though the uterine contents are growing at a rapidly accelerating rate. This is, therefore, a period of *uterine stretching.* These three stages of uterine accommodation—preparation, growth, and stretching—are characteristic of all uteri thus far studied. The characteristics of the three periods of uterine accommodation vary, however, from one species to another.

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* Further discussion of some of the points raised in this paper, together with bibliographical references, appear in S. R. M. Reynolds' *Physiology of the Uterus.* Paul B. Hoeber Publishing Co., New York, N. Y., 1949.

At one extreme, for example, the hamster exhibits a period of stretching that lasts but one day and a half; at the other extreme, the guinea pig has a period of uterine stretching that continues for more than three weeks. Between these two extremes, the period of stretching in the rat lasts four days; in the rabbit, eight. Allowing for differences in the length of gestation periods in these different forms, this means that the proportion of pregnancy occupied by the period of uterine stretching varies in an orderly way among the several species.

Just as the development of the products of conception is related to the period of uterine stretching, so also is uterine growth related to the products of conception. When, for example, pregnancy is limited to one horn of the uterus duplex, only the horn containing the products of conception grows at first. The sterile horn remains unchanged in size. The reason for this is that the interplay of hormones and uterine distention is such that there is localized growth of the uterus as a function of local distention. Because this is true we can comprehend that when, in a litter-bearing animal, the size of the litter is small, the amount of uterine growth is also small. Conversely, when the litter is large, uterine growth is large, too. This factor, that the amount of uterine growth in mid-pregnancy is affected by the size of the uterine contents, obviously plays a role in determining the amount of stretching the uterus is capable of undergoing in the last part of pregnancy during the period of uterine stretching. Thus, the size of a litter affects the size of the uterus and the size of the uterus affects the extent to which the uterine contents can stretch the uterus in the last part of pregnancy. Both uterus and its contents, therefore, interact to affect the span of gestation and the maturity of the offspring.

Uterine Shape During Gestation

There is another very important way in which the conceptus influences the uterus. This is the role that the shape of the conceptus plays in the course of uterine accommodation. Soon after implantation, the accumulation of fluid in the tiny conceptus forces the uterus to bulge locally in order to accommodate it. This shape is, for all practical purposes, spheroidal. As the conceptus enlarges, the area of distention becomes frankly spheroidal, and the process continues until, at a specific time critical for each species, a maximum radius is attained. Then, within a space of time marked in hours, the conceptus elongates. Thereafter it grows as a cylinder. This change from one pattern of enlargement to another has been called *uterine conversion*. If the uterus is that of a litter-bearing animal containing more than one conceptus, the conceptuses come to lie end to end after uterine conversion. In this way, further uterine enlargement is brought about through an increase in length, with no further significant increase in diameter.

These observations are doubtless familiar facts to those acquainted with gestation in domestic and laboratory animals. Certain physiological consequences inherent in these facts, however, are not generally known, and these I wish to clarify. They relate to the efficiency of the flow of maternal blood through the uterine wall as the uterus inevitably changes its shape to accommodate its contents.

Uterine circulation. Three different types of study have shown that maternal blood flows abundantly throughout the uterine wall during the early and middle periods of spheroidal uterine enlargement. As the conceptus approaches its maximum spheroidal size, however, the uterine tissues begin to be stretched, and tension develops within them. The tension builds up to an amount detrimental to maternal blood flow. In fact, in the rabbit on the twenty-second day of gestation, when the conversion from spheroidal to uterine enlargement occurs, the tension is so great that an ischemia amounting almost to complete circulatory stasis takes place, except in the neighborhood of the placenta. In fact, owing to the very special shape of the uterus, blood flow is assured to the placenta at this time.

Conversion from sphere to cylinder, which requires only a few hours, as mentioned, heralds a release of uterine tissue tension and, with this, there is re-establishment of the maternal blood flow throughout the uterus. In the final period of uterine accommodation, that of uterine stretching, one might expect a continuing diminution of the flow of blood. Nature has contrived, however, by means of a special arrangement of the maternal blood vessels within the uterine wall, to minimize the effect of stretching upon the blood vessels at this time. Ultimately, however, near the end of the period of uterine stretching when the fetus is gaining weight at its most rapid rate, a second period of uterine ischemia ensues. The flow wanes rapidly. What is the significance of these two periods of uterine ischemia in the course of uterine accommodation?

The first period of ischemia is overcome when the uterus changes shape; the second period of uterine ischemia is overcome by parturition. Proper uterine accommodation, therefore, serves to minimize both of these hazards through timely and adequate change of shape on the part of the conceptus. Clearly, any deviation from the normal course of events must necessarily jeopardize the welfare of the unborn offspring, possibly even resulting in its death.

Unity of the Uterine Accommodation Process

I have discussed at some length the uterine function in the accommodation of the products of conception during the course of gestation. I have described its development in terms of uterine enlargement and change of uterine shap and with respect to the flow of maternal blood through the tissues of the uteru We have seen how the growing fetus and its associated structures affect eac of these factors. This, then, is the underlying theme of this paper, namely that one should not think of the fetus as an entity dependent only on it placental circulation, but rather, along with the uterus, as part of a functiona unit in which the uterus, the size and welfare of the offspring, and the course o gestation are continually interrelated and interdependent.

How does this concept bear upon those aspects of the reproductive processe with which students of reproduction, animal husbandrymen, and veterinarian are concerned? Specifically, how does it relate to the size and number o offspring in a litter? To intrauterine death? To the span of gestation, th maturity of the young at birth, and to the onset of labor? In the light of th continuity of the accommodation process throughout the span of gestation, w shall examine briefly each of these aspects of pregnancy. To do so wil enhance our understanding of the extraordinary simplicity of an otherwis seemingly complex and baffling group of unrelated physiological activities.

Fetal size at birth. The size of a litter is an important economic factor in th breeding of all domestic animals. Experience shows that in some species larg litters are not desirable because of a high rate of loss among the newborn. I others, large litters are economically desirable because of the number of youn that do survive. In what way does the gestation mechanism bear on th question of litter size?

Since the work of Hammond, it is axiomatic that for inbred strains of rabbit the smaller the number of young in a litter, the larger the individuals and th more mature they are at birth, as well. Conversely, the larger the litter, th smaller the individuals and the less mature they are at birth. This is true i many domestic animals—so much so that in the mare, live multiple births ar virtually never seen, for the young are born at too early a stage of pregnancy t survive. This fixed tendency should be borne in mind by those who bree animals for meat, hide, or head.

What is the physiology behind this factor? An insight is gained when w examine total conceptus weight in large litters of different sizes. We find that in small litters having larger fetuses near term, the combined weight of al fetuses approaches that of the total conceptus weight in large litters made up o smaller individuals. There is, accordingly, less disproportion than one migh think, as far as the uterus-fetus relationship is concerned. For example, rabbi litters averaging 3.5 young to a litter have a combined weight of 280 gm., o 8 gm. of uterus per fetus, while litters of 6 young total 484 gm., or 8.06 gm. o uterus per fetus.

Uteri containing each size of litter vary. In both smaller and larger litter the uterus increases the same amount in weight, from 2 to 3 gm. at the outset to about 18 gm. by the sixteenth day of gestation. Thereafter, the uterus increases by about another 10 gm. in the next fifteen days. In the case of larger litters having the larger uterine load, the uterus makes one final spurt o growth in the last three days of pregnancy. Less of a spurt of growth occurs in smaller litters. This is the situation as known in the rabbit.

On the surface, this is a very baffling situation, for it shows that the uterus responds to its load by greater growth, but it does not tell us what the two different patterns of uterine growth might have in common to provide a mechanism for the onset of labor. We find the common denominator in one further correlation. It exists as a function relating the weight of the uterus to its contents. When, for each series of data, one divides the weight of the uterus by the weight of its contents, a pair of curves may be plotted from the data showing that, as gestation comes to an end, the amount of distention in the case of both small and large litters is about equal. One could hardly visualize a more striking mechanism than this one in which, despite variations in the size of the litter, of the uterus, and of the individuals within the litter, a very constant relation exists between the uterus, on the one hand, and the size of its contents, on the other. Clearly, the whole business of uterine accommodation in the latter part of gestation seems to be concerned with the development and maintenance of this relation, for reasons not yet known to us. It is probably governed by the tissue tensions, the muscle thickness necessary to develop the power for labor, and the hormonal stimuli that function at this time. In any case, it is clear that, as far as the physical relations with respect to small or large litters are concerned, the same ultimate functional state of the reproductive tract is achieved in both instances. This role of distention, although long appreciated as a factor in the gestation mechanism, has never been defined with the unity it has in this definition in relation to uterine accommodation, yet distention appears to be a critical factor in the mechanism, so regular is it in form and in magnitude.

Before leaving the question of fetal size at birth, it would be remiss not to refer to two further factors that influence the size of the offspring at birth. One is dietary, the other genetic.

Stated briefly, severe caloric restriction has no effect on the size of the young at birth, unless the restriction exists in the last stage of pregnancy. It then becomes a determining factor and results in fully developed offspring that are smaller than normal. Restriction early in pregnancy with full diet in the latter part results in the birth of young of normal size. How the uterus itself is affected under these circumstances is not known but, in view of the relationship existing between this organ and litter size generally, I venture to predict that the role of caloric intake will be found to exert itself through the mechanism of uterine accommodation already described.

A genetic factor operating through the mother likewise may be demonstrated as affecting the size of offspring. For example, a Yorkshire stallion bred to a Shetland mare will produce a foal always the size of the pure-bred Shetland foal. A foal of such size is compatible with the small pelvic outlet of the mother. A Shetland stallion, on the other hand, bred to a Yorkshire mare will produce a large Yorkshire-type foal. This indicates that something in the maternal uterine environment plays an as yet undefined role in this limitation of size of the offspring. One would do well, I believe, to investigate th uterus in such situations, instead of simply concluding that genes are responsible. They do play a basic role in inheritance, but the physiologist must concern himself with the mileiu in and through which genes work.

We may accept as a first principle, I think, that the uterus affects the siz of offspring, and that the size of the offspring affects the uterus. The tw entities are mutually interdependent throughout the course of gestation.

Fetal Maturity

Maturity within the species. One of the lessons uterine physiology taugh long ago is that, the larger the litter, the less mature are its individuals a birth and, conversely, the smaller the litter the larger and more mature are th young when born. Small rabbit pups are always immature, large ones ar always quite mature. Why should this be so? The answer is related to th fact that large litters are commonly born a day or two before their less numerou and larger litter-cousins.

Hammond and Wishart have found that the answer to the problem is rathe simple, and a neat physiological mechanism. By using inbred strains o rabbits, these investigators produced one variety that tended to parturition o the thirty-first day of pregnancy, and another variety that came to term on th thirty-third day. The former are less mature at birth and smaller than are th offspring resulting from a pregnancy of thirty-three days' duration. In th latter variety, there is a genetic tendency for the corpora lutea of gestation t function for a slightly longer time in does exhibiting longer gestation periods. It is clear that, when the last period of uterine accommodation, which involve extensive fetal growth, is extended by the presence of corpora lutea that function for a day or two longer, the fetuses are assured of a favorable environment for two important days. In the case of single strains, the large litters tend t come to term at an earlier time than small litters. This is without doubt related, as we have seen, to the fact that they impose sufficient load upon th uterus to develop the critical distention coefficient at an earlier time in gestation than do their larger cousins of smaller litters. In this way, they are born both smaller and less mature than they would be if they were to remain *in utero* for a day or two longer.

Comparative fetal maturity at birth. When we consider the relative degree of maturity among the several species, we observe the same kind of mechanism at work. For the hamster, the period of uterine stretching is a mere 1.5 days; for the rat, 4 days; for the rabbit, 8 days; and for the guinea pig, about 30 days. It is clear to anyone familiar with these species that this series from hamsters to guinea pigs constitutes one of increasing maturity at birth. This progression of maturity can be demonstrated with respect to such diverse phenomena as the degree of ossification, the resistance of the newborn to anoxia, and the attainment of control of body temperature.

We see, therefore, that whether within or among the species fetal maturity is related to the phases of uterine accommodation, and especially to the period of uterine stretching that represents the accommodation by the uterus of a rapidly maturing fetus. We may say as a guiding rule that the greater the proportion of the period of pregnancy that a fetus spends within the uterus after conversion of the pattern of uterine enlargement from sphere to cylinder, the more mature will the offspring be at birth. This shows a direct association between the conceptus and the uterus in the mechanism of gestation.

Fetal Death

We may expect to find a relationship between fetal death and any abnormal tendency in accommodation that is comparable to the dependence of the viability of the fetus on the processes of uterine accommodation. This would be pronounced, perhaps, at the time of uterine conversion, for it is then, as we have seen, at that potentially critical time, that profound ischemia takes place as a necessary phase of adjustment of the uterus to its contents.

In order to assess this possibility, counts of resorbing fetuses have been made in a series of rabbits in which the number of dead fetuses was correlated with the phases of uterine accommodation. After implantation and until the sixteenth day, the number of resorbing fetuses is negligible. As the maximum spheroidal size of the conceptus is approached, however, we find an increase in the number of deaths, which continues until the twenty-fourth day of gestation, by which time the process of conversion is over. The next four days show no further increase in the number of dead fetuses but, toward the end of gestation after the second period of uterine ischemia commences, there is a second very abrupt increase in the number of fetal deaths.

The fact that there actually is an increased incidence of fetal deaths in rabbit litters related to the development of each period of uterine ischemia suggests that fetal death is caused by a blood flow insufficient to sustain some of the fetuses. The observation substantiates but does not prove that faulty uterine accommodation is related to fetal death at these times. It is a reasonable tentative conclusion, however, based on observed facts, and it reveals again that the interdependence of the uterus and its contents is indeed an intimate one.

The Span of Gestation and the Onset of Labor

One of the mysteries of the ages is the tendency for gestation to last for fixed engths of time among the several species of animals. There are variations, as n all biological phenomena, but so fixed is the mean duration of pregnancy that physicians as well as breeders of all animals look upon a fixed gestation period as characteristic of a species or breed. In seeking for a factor that governs this, many investigators reveal a tendency to reduce a complicated process to a very simple basis. They speak of "*the* cause of the onset of labor." It is widely believed that some factor acts at just the proper time to pull the rigger of the parturition process. We shall see that this is not so. It is no more possible for this to happen in parturition than it is for a play to have a dramatic climax without a careful build-up of the dramatic action leading to it.

The circumstances leading up to the parturition mechanism have already been outlined, showing that the uterine contents grow at an accelerating pace whi the uterus approaches a limiting degree of distention. Inherent in this proce is a decline in circulatory function, reflected in a lessened flow of matern blood in the uterine wall in a diminishing rate of transfer of water and certa dissolved substances across the placenta.

During the last week of pregnancy in the rabbit other changes also occ that herald impending labor. Most prominent of these is an accelerating i crease in myometrial irritability, which is greatest within the last two days pregnancy. This irritability may be demonstrated by an increased sensitivit of the myometrium to certain hormones, and to mechanical, electrical, ar pharmacological stimulation, as well as by an increasingly efficient pattern uterine contractions. At the same time, other factors also come into pla Increasing uterine distention increases the resting tissue tension, and this favo a greater force of contractions. Behind these more evident changes is the fa that near the end of gestation actomyosin, the contractile protein of muscl increases in quantity and improves in quality, which facilitates forceful co traction. Taken together, all these factors develop the capacity for labor an provide the coordination of myometrial contractility that makes possib effective labor.

Presiding over all the forenamed changes are the hormones generated by th ovary and placenta. These hormones play a key role. Progesterone, at lea in certain species, inhibits the labor mechanisms until its influence is effectivel diminished and the labor process can begin. Estrogens affect not only th growth of the uterus but also its contractility. Many workers have questio as to how the relationship between estrogen and progesterone necessary fo labor comes about, and what the pattern of hormone levels in the blood i There is no question, however, concerning the fact of the qualitative actions the ovarian and placental hormones. Until the pregnancy-stabilizing effect progesterone is withdrawn, there will be no labor. In some species this hormon is derived entirely from the ovary throughout pregnancy, and in others fro the ovary and placenta. Estrogen, on the other hand, increases in amoun and in effective action as term nears. Without the proper action and balanc of these hormones, labor would not occur and the fetus would die.

The labor process thus displays a complex group of related functions i which factors tending to stabilize pregnancy are gradually overcome by other serving to bring gestation to an end. Labor, accordingly, is the result of gradual, accelerating convergence of a number of factors—some morphological some physiological, some biochemical, and some physical—all of which converg like a beam of light to a functional focal point, namely, the end of gestatio and the initiation of labor.

I do not doubt that the onset of labor requires not only all the change described above, but others as well. Failure of one or more of these factors t develop properly will give rise to abnormal or delayed labor. Premature con vergence to a critical point may precipitate a premature labor, even at th expense of a mature fetus.

Conclusion

Several aspects of uterine physiology have been discussed. We have con-
dered the pattern of growth of the fetus and uterus, as well as the local and
ndocrine factors that affect the relation between the uterus and its contents.
e have noted the orderly change of shape of the uterus during the several
ages of uterine accommodation, and how at times this aids and at other times
inders the uterine circulation. The fetus must undergo extreme physiological
terine ischemia, which it may not survive. Fetal maturity at birth has also
een shown to be related to the mechanism of uterine accommodation. Finally,
e have noted that, as the last phase of uterine accommodation becomes estab-
hed, the contents of the uterus outgrow the confines of an increasingly un-
avorable uterine environment. Either parturition or fetal death is the in-
vitable climax of this related series of events.

Due to my efforts to simplify a very complicated group of processes, it may
ppear to some that the factors which control the span of gestation and the
nset of labor are now known in all essential details. There is much more for
s to learn, however. I am certain that scholars with a knowledge of gesta-
ional processes in a wide variety of species will have much of vital importance
o add. We need to know what governs the all-important rise and fall of
ritical hormone production and effectiveness, and what makes the uterus
ollow its organized patterns of accommodation and contractility.

IMPLANTATION

By Bent G. Böving

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The implantation of the human ovum may be described by a sequence anatomical stages of approximately known age (TABLE 1). From the time ovulation it is about 1 week until the blastocyst attaches to the uterus, weeks until the basic plan of the placental vasculature is established, and weeks until the fetal placental circulation begins (Hertig and Rock, 1951 The period of implantation, broadly construed, might include all of this excep the first 3 days or so when the egg is in the tube. An advantage of this vie is that it includes sufficient specimens to tell a connected story (TABLE 1) an in so doing, to reveal the significance of certain gaps. One of the most seriou of the gaps appears between the free blastocyst stage—4½ days after ovulatio –and the stage when the blastocyst is below the epithelium—7½ days—(Herti and Rock, 1951; Hertig *et al.*, 1954). Since the blastocyst's passage from th uterine lumen through the epithelium to the submucosa must occur durin this time, it is impossible, in the strict sense, to describe the period of implantation.

This is a matter of regret but not of despair, for if the gap is not filled b new discoveries of young human embryos, it seems quite possible to do s eventually; it will be done with material from the chimpanzee. The chimpanzee blastocyst lodges beneath the uterine epithelium, as does the human and its placentation resembles that of the human in its earliest known specime —10½ days—(Heuser, 1940), as well as later (Wislocki, 1933).

In the meantime, a description of human implantation must necessarily b either frankly incomplete or frankly speculative in some essential parts. choose the latter alternative, in the form of a comparative approach, for th sake of perspective and the possibility of experimentation. Thus, the unknow period can be scouted from a functional as well as a structural point of view.

The early development and implantation of the macaque (*Macaca mulatta* have been studied carefully (Wislocki and Streeter, 1938; Heuser and Streeter 1941). This animal resembles the human in having a menstrual cycle length o about 28 days and in having a spontaneous single ovulation near the middle o most cycles (Hartman, 1932, 1933). The blastocyst enters the uterus about days after ovulation (Lewis and Hartman, 1941), about the same time as the human and, like it, is covered by a zona pellucida. It implants somewhat later than the human, since it can be flushed out on the eighth and ninth days after ovulation (Heuser and Streeter, 1941), whereas the human is thought to implant about 6 days after ovulation (Hertig and Rock, 1951). An important difference is that the macaque blastocyst does not lodge beneath the uterine epithelium, although it does send trophoblastic attachments through the epithelium. The establishment of a second placenta on the opposite uterine wall usually follows (Wislocki and Streeter, 1938), but the percentage of exceptions is substantial (Ramsey, 1954). Thus, the macaque and human are most alike

TABLE 1
EVENTS RELATED TO HUMAN IMPLANTATION

Days after ovulation	Horizon	Characteristics
	I	Zygote, one cell; in tube
2–4	II	First cleavage to morula; transported through tube into uterus
4–7	III	Blastocyst, segmentation cavity appears and expands; in uterus, transported to attachment site
7–10	IV	Attaching ovum (unknown but inferred: blastocyst adheres to uterus, trophoblast penetrates uterine epithelium, blastocyst passes through epithelium); blastocyst cavity is collapsed after blastocyst has passed through epithelium; trophoblast grows and, in older specimens, has lacunae.
10–13	V	Imbedded ovum, covered by uterine epithelium; chorionic cavity distended, no villi yet
13–15	VI	Beginning mesodermal villi

The original definition of these early horizons (Streeter, 1942) has been supplemented nd adapted for the present purpose. Advice from C. H. Heuser is gratefully acknowledged.

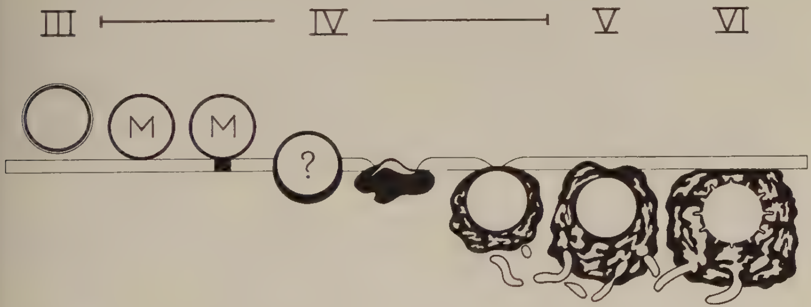

FIGURE 1. The implantation of the human ovum, illustrated here, is outlined in TABLE 1. he serious gap, comprising the last part of Horizon III and the first part of Horizon IV, entatively is bridged by two stages known in the macaque (M) and one hypothetical stage). The zona pellucida is drawn only in the stage where it is known to be present; the possiility of a longer persistence is discussed in the text.

the events prior to the unknown portion of human implantation; they are nlike after that period. Since the divergence must begin during the unknown eriod, any estimation of the events of that period in the human from the events f a corresponding period in the macaque must be tentative. This applies to e following account and the accompanying diagram (FIGURE 1).

The human blastocyst enters the uterus about 3 days after ovulation (Hertig *al.*, 1954). It is transported to the site of attachment during the next 3 ays. Judging from the macaque (FIGURE 2), it is probably covered by the na pellucida during most of this period (Streeter, 1938). The human zona llucida appears to disintegrate earlier than the monkey's and regionally rather an uniformly (Hertig *et al.*, 1954), although this may be an artifact. Judging

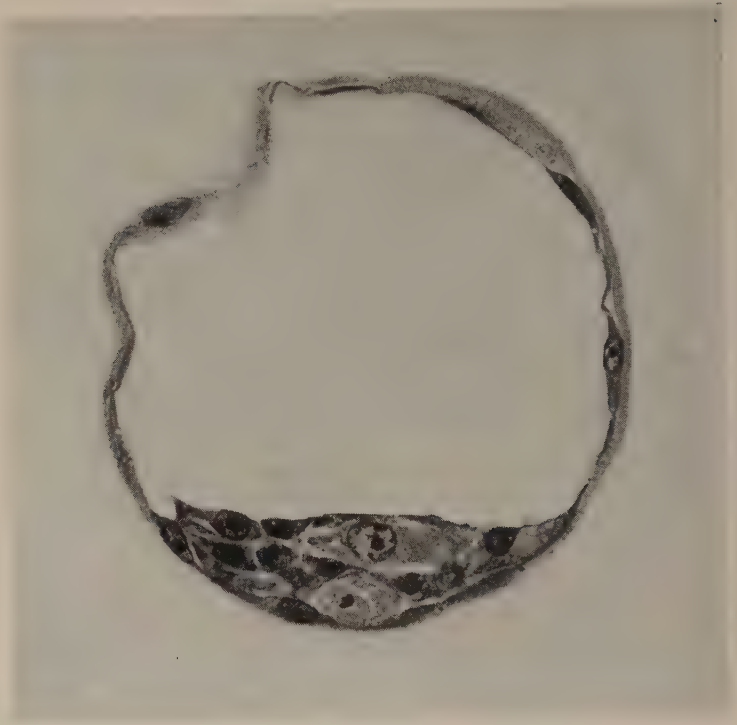

FIGURE 2. The macaque blastocyst (C-614), about 8 days after ovulation, is envelope by a zona pellucida that is more degenerated at the embryonic and abembryonic poles tha equatorially. ×500. (This photograph, from Heuser and Streeter, 1941, is republishe with the permission of C. H. Heuser and the Carnegie Institution of Washington.)

from the monkey (FIGURE 3), and allowing for time differences, human attachment is believed to begin with trophoblastic adhesion to the uterine epithelium and penetration of it at about 6 days after ovulation (Hertig and Rock, 1951). The completely mysterious passage of the human blastocyst through th epithelium has already occurred in the earliest implanted specimen (FIGURE 4) whose age is estimated at 7½ days. A temporary loss of blastocyst turgo seems a likely accompaniment and possible mechanism of the passage, sinc this blastocyst is collapsed, whereas younger and older ones are not. Alread in this specimen the trophoblast has begun to spread into the submucosa b proliferation and invasion. This proliferation advances most rapidly in th regions away from the epithelium and, during the next few days, the epitheliu closes the gap left by the blastocyst's passage (FIGURE 5). In its proliferatio and invasion, the trophoblast envelops blood vessels. At the same time, abou 9 or 10 days after ovulation, lacunae or spaces develop in the trophoblast (Hertig, 1951). Walls of the engulfed blood vessels disappear, and intervenin trophoblast yields in such a way that maternal blood enters the lacunae (FIGUR

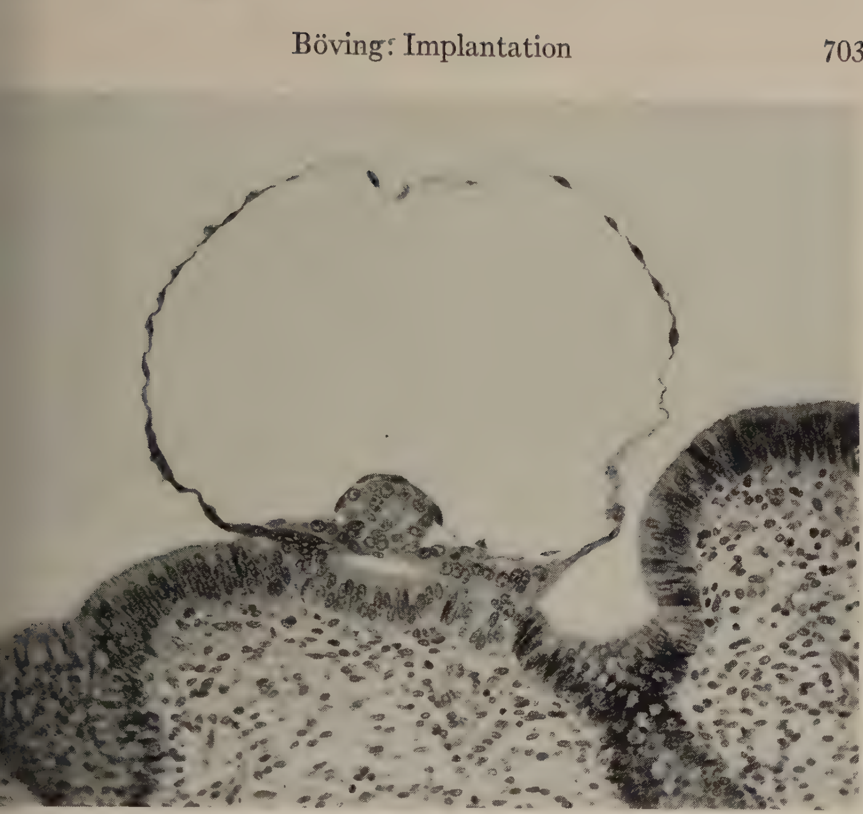

Figure 3. The macaque blastocyst (C-610), 8 to 9 days after ovulation, adheres to the terine epithelium. The epithelium is injured and partially penetrated by trophoblast. <200. (This photograph, from Heuser and Streeter, 1941, is republished with the permision of C. H. Heuser and the Carnegie Institution of Washington.)

). This development late in Horizon IV (table 1) establishes the basic plan f the maternal circulation of the placenta, namely, maternal blood in a space ned by trophoblast (the intervillous space is derived from the lacunae). The asic plan of the fetal circulation becomes evident in Horizon VI, about 2 weeks fter ovulation. Villi are formed when mesodermal cores, the predecessors of etal vasculature, enter trophoblastic projections into the lacunae (figure 1). n the next 2 days (Horizon VII) the villi branch and, at 3 weeks (Horizon X), hen blood begins to flow in their vessels, the placenta may be considered nctionally established (Wislocki and Streeter, 1938; Streeter, 1938; Heuser nd Streeter, 1941; Hertig and Rock, 1941, 1945, 1949, 1951; Krafka, 1941).

ıe next 2 days (Horizon VII) the villi branch and, at 3 weeks (Horizon X),

A description by scheduled anatomical stages, such as the preceding, is posble only because the events have a consistent order in time and space. This nplies rigid control mechanisms. In seeking these mechanisms there is some dvantage in giving stages rather mechanical labels—such as blastocyst transort, grasp, shedding of zona, and adhesion; trophoblastic invasion of endo-

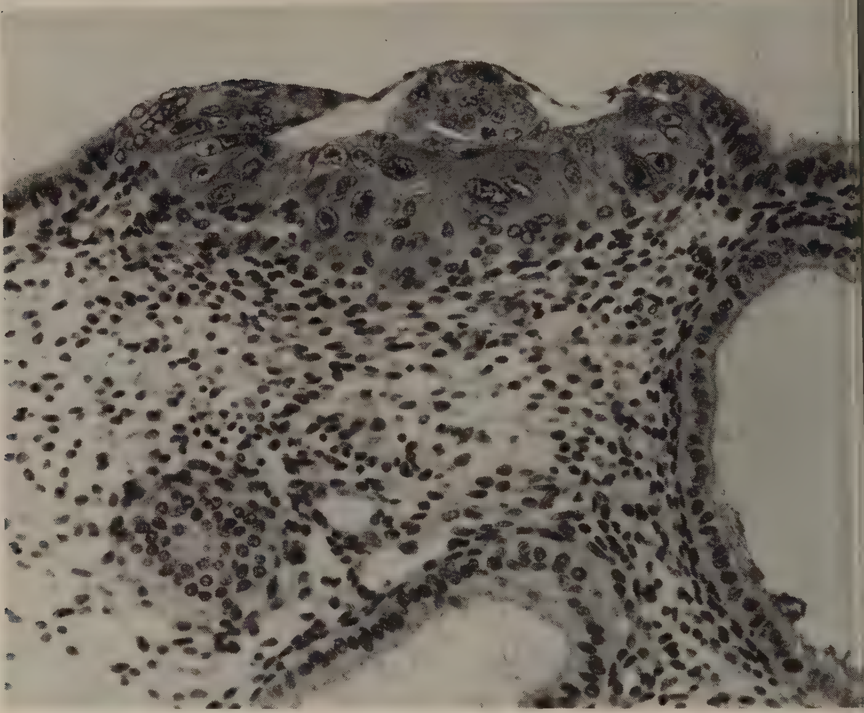

FIGURE 4. The human ovum (8020), Horizon IV about 7 or 8 days after ovulation, ha passed through the uterine epithelium and lodged in the submucosa. The collapse of th blastocyst is striking. Epithelium in contact with trophoblast has been injured. ×25 (This photograph, from Hertig and Rock, 1945, is republished with the permission of th authors and the Carnegie Institution of Washington.)

metrium; and passage of the blastocyst through the epithelium. These will b considered in turn.

Transport

In monkeys, 41 of 44 implantations were centrally located; 20 were posterio and 21 were anterior. The 1 cervical and 2 lateral exceptions were atypica ova (Heuser and Streeter, 1941). These authors pointed out that this consistency in the site of implantation, which resembles the human, could not b explained by preformed implantation sites randomly arrived at, for no suc sites were discernible before implantation. Nor were cilia to be considere responsible, because "directional force of such precision is not characteristic o ciliary movements, and furthermore the surface epithelium of the uterus of the macaque is not ciliated except for occasional isolated cells, so far as the writer of this account have been able to learn." They were inclined to agree wit Hartman (1939) that the blastocyst is consistently carried to its centrally

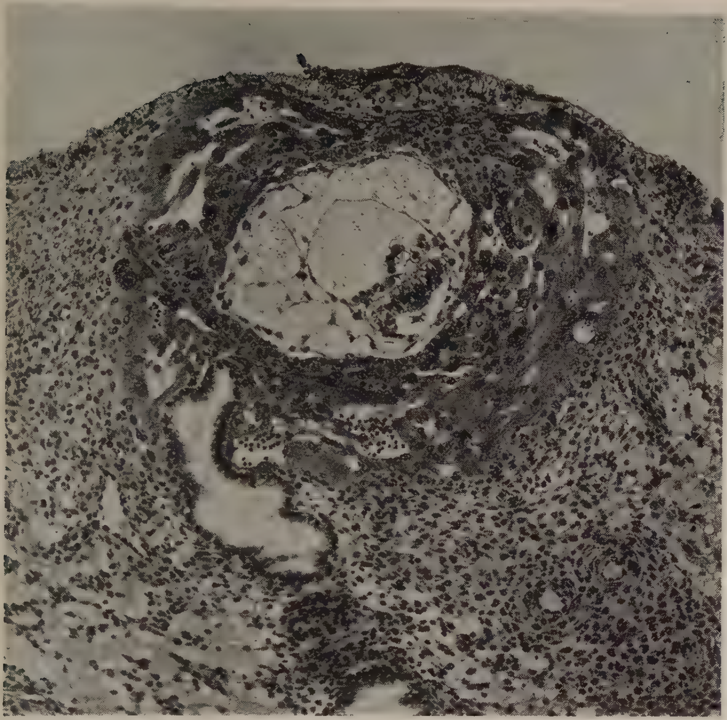

FIGURE 5. The human ovum (7699), late Horizon IV about 11 days after ovulation, has
xpanded and here appears to be covered by epithelium. Other sections show that the cover
not quite complete. The trophoblast has developed lacunae, has enveloped some blood
essels (at right), and is growing around a gland (lower left). It touches the uterine epithelium
hich is covering the ovum, but does not injure it. ×100. (This photograph, from Hertig
nd Rock, 1941, is republished with the permission of the authors and of the Carnegie Institu-
on of Washington.)

ocated implantation site by uterine muscle, probably acting through eddy
urrents.

It is agreed that few cilia are seen in the earliest implanted monkey specimen
C-520); none were found in a cursory study of the slightly older C-560. It
ust be admitted, however, that the hematoxylin and eosin stain on them is
ot favorable either for seeing the cilia or differentiating them from the mucus
resent. The azan stain renders cilia red and the mucus blue. It was applied
o sections of a fistulous uterus from a nonpregnant macaque (884) killed on
e nineteenth cycle day. Since the endometrium was progestational, the
ycle is presumed to have been ovulatory, and the age of the specimen is
stimated to be 5 or 6 days after ovulation. That would be the period of
astocyst transport if the animal had been pregnant. Cilia were present
n about 5 to 10 per cent of the cells of the surface epithelium. Most of the
liated cells were isolated, but a considerable number were in pairs. Blastocyst

transport by cilia cannot be unequivocally ruled out by their complete absence yet their mere presence on a few cells cannot be considered sufficient evidence for this or any other particular function. This small extension of the reasonin of Heuser and Streeter and Hartman still leaves the blastocyst-transportin role of cilia undetermined but doubted, and the role of muscle favored but ur proved. The best evidence for the latter is the appropriate cyclic variation i tubal and uterine contraction (Corner, 1923).

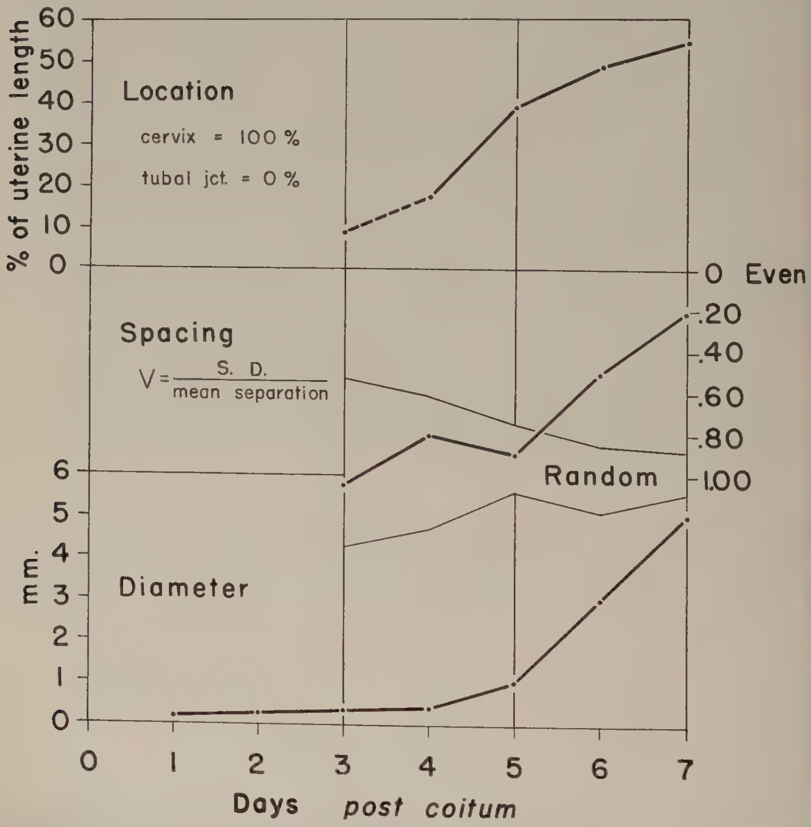

FIGURE 6. Three synchronous graphs describe the relationship of rabbit blastocyst diameter and distribution (location and spacing). Spacing, measured by V, becomes rapidly more even between 5 and 7 days *post coitum*, just when blastocyst diameter increases rapidly. This suggests that blastocyst expansion is the stimulus for even spacing, and similar spacing of models according to their size confirms it. In addition, one perceives that blastocysts are carried toward the cervix most rapidly during the 3- to 5-day period when they are farthest from it, as well as small. Transport is slower during the 5- to 7-day period when they are closer as well as larger. This and the reasons mentioned in the text suggest that propulsion arises from the cervical as well as the tubal end of the uterine horn. Such retrograde propulsion may retard blastocyst transport, and the concurrent blastocyst expansion may or may not do so too. [The data for blastocyst diameter at 1 and 2 days were provided by G. S. Greenwald prior to his recent publication of them (Greenwald, 1957), and his data for 3 days were used to confirm my own.

Blastocyst transport by uterine muscle is more easily studied in rabbits than n primates, because quantitative and experimental methods may be more eadily applied. Not only are rabbits available in quantity, but each animal provides numerous specimens of precisely predetermined age, all in a row within a long tubular uterus. Moreover, rabbit blastocysts are large, reaching 5 mm., and they are easily observed and manipulated.

About 20 uterine cornua per day, from 3 through 7 days *post coitum*, were opened logitudinally, and the location of the blastocyts within each were neasured and converted into the percentage of the distance from the utero-ubal junction to the internal cervical os. Several statistics were calculated hat reflected the progress of the blastocysts along the uterus, their increasing eparation from each other, and the evenness of their spacing (Böving, 1956). From 3 to 5 days *post coitum* they were carried progressively down the uterus n a random arrangement. The normal net progress then averaged about 1.2 nm. per hour; the fastest blastocyst of the 617 observed moved about 6.2 mm. per hour between 3 and 4 days *post coitum*. From 5 to 7 days *post coitum* he average rate of progression was slower (0.6 mm./hr.), and the blastocysts gradually became more evenly spaced. The changes in rate and regularity coincided with the rapid increase in blastocyst diameter, which began at 5 days *post coitum* (FIGURE 6). This blastocyst expansion has been shown to depend on progesterone (Corner, 1928). Progesterone also causes rabbit uterine muscle *in vitro* to have high irritability (Csapo and Corner, 1951), with unimpaired ability to contract locally, but with decreased ability to conduct propagated contractions along the length of the uterus (Csapo, 1955). A lower efficiency n producing longitudinal tension results (Csapo, 1954). Very small amounts of estrogen alter the typical progesterone-dominated muscle action (Corner and Csapo, 1953). Muscle behavior *in vitro* during estrogen-free progesterone domination imitates the decreased forcefulness and propagation of contractions observed (Markee, 1944) *in vivo* after 5 days *post coitum*.

The preceding facts may be related by the hypothesis that blastocyst transport is a random scattering while the blastocysts are small, but that progesterone causes their expansion and, in the absence of estrogen, conditions the uterus so that contractions arise from each end of the uterus and wherever it is timulated by a blastocyst above a certain size (FIGURE 7, reaction 1). These ontractions are propagated along the uterus in both directions, but they lose propulsive efficiency with increasing distance from each source. In that way, ach blastocyst may cause uterine repulsion of its neighbors, while it remains unaffected by the contractions stimulated by itself. Since the repulsion is mutual nd weakens with distance, one may expect the blastocysts to be moved apart ntil the repulsions are cancelled out by equal (maximum) separation between ll the blastocysts in the cornu (Böving, 1956).

The blastocysts continue to expand after they are approximately evenly paced and, after a certain size is reached, they can no longer be moved along he uterus. Soon the antimesometrial muscle overlying each blastocyst loses one, and a dome is formed in which a blastocyst lies (FIGURE 7, reaction 2). The circular muscle ahead of and behind the blastocyst forms thickened bands

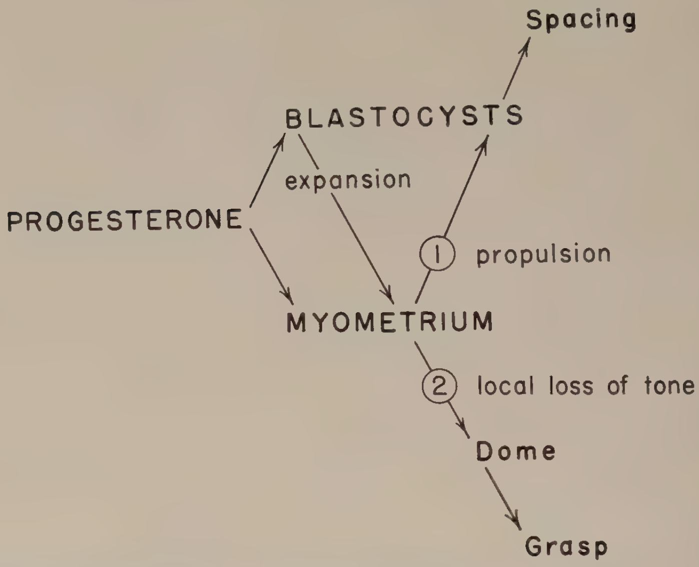

FIGURE 7. This diagram suggests a progesterone-conditioned mechanism whereby blastocysts are carried to their sites of implantation and held there.

(FIGURE 9). It is believed that this incomplete sphincter, perhaps aided by the contours of the lateral folds of the endometrium, is responsible for the muscular "grasp" demonstrated *in vivo* (Böving, 1952, 1954). This grasp, in effect a muscular stage of attachment, holds the blastocyst antimesometrially and insures that the succeeding adhesive and invasive stages of attachment will be consistently antimesometrial.

The quantitative analysis of blastocyst location in normal rabbits defined the limits within which one might expect to find 95 per cent of normally located blastocysts (Böving, 1956). At 6 and 7 days, this normal range may be defined even for individual blastocysts if the number per horn is known and agrees with the corpus luteum count. For experimental purposes it is convenient to have some of this information expanded into a table of normal location at 7 days *post coitum*, the time of attachment (TABLE 2). Thus, it may be determined readily if blastocysts, following various experimental treatments, have a location significantly different from normal ($P = 0.05$).

Extremely small doses of estradiol benzoate, comparable to those shown to alter typical progesterone-dominated myometrial behavior *in vitro* (Corner and Csapo, 1953), caused abnormal spacing if given just before the onset of spacing (Greenwald, 1957). Reasoning backward, this shows that essentially estrogen-free progesterone domination of the myometrium is normal and essential for even spacing. Since the endocrine control operates similarly *in vitro* and *in vivo*, it shows also that the uterine contractions concerned are myogenic rather than neurogenic.

TABLE 2

NORMAL RABBIT BLASTOCYST LOCATION AT 7 DAYS *Post coitum*

N												
1	31–77											
2	21–53	54–87										
3	15–43	40–68	65–93									
4	12–36	32–56	52–76	72–96								
5	9–32	26–49	42–65	59–82	76–99							
6	7–29	22–43	36–58	49–71	65–86	79–100						
7	6–27	18–39	31–52	43–64	56–77	68–89	81–102					
8	5–25	16–36	27–47	38–58	49–70	60–81	72–92	83–103				
9	4–24	14–34	24–44	34–54	44–64	54–74	64–84	74–94	84–104			
10	3–23	13–32	21–42	30–50	40–59	49–68	58–77	67–86	76–95	85–105		
11	3–22	11–30	19–38	28–47	36–55	44–63	53–72	61–80	69–88	78–97	86–105	
12	3–21	10–29	17–37	25–44	33–52	40–60	48–67	56–75	63–83	71–90	79–98	87–106

The left column, N, indicates the number of blastocysts in the cornu. The ranges of successive blastocysts in that cornu are read horizontally. The range is expressed in per cent of the distance from the uterotubal junction (zero) to the internal cervical os. The ranges reflect the mean location, the evenness of spacing, and the variability of both. These criteria are sufficient to predict the range of 95 per cent of blastocysts in normal rabbits. Thus, a blastocyst or model in an experiment is not significantly different from normal ($P < 0.05$) if it is within the range. The derivation of the range is given elsewhere (Böving, 1956) together with data for testing normalcy of distribution by the separate criteria and on other days of gestation.

The initiation of even spacing by blastocyst expansion is suggested but not proved by their synchrony and by incidents such as the "ignoring" of an abnormally small blastocyst (FIGURE 8). We must decide whether the stimulation of spacing contractions depends on a physical property of the blastocyst, such as size or weight, or whether it can be explained by a biological or chemical mechanism that just happens to operate at the same time as the expansion. For this purpose, solid glass spheres 3.2 mm. in diameter were placed in one uterine horn at 2¾ days *post coitum.* Ligation prevented blastocysts from entering this horn; the contralateral horn was an intact control (sham operation was not used, but perhaps should have been). The diameter of the models was the same as normal blastocysts at 6 days *post coitum*; it had been found that larger models were not well propelled. At autopsy 7¼ days *post coitum,* 5 of the 6 models were within normal limits for 6 days. The one exception was out of bounds by only 0.5 per cent of the uterine length, and this is within the range of technical error. The controls had an average diameter of 2.9 mm. This was almost identical with the models and more appropriate to 6 days than to their actual age of 7¼ days. Of the 4 blastocysts, 3 were within normal limits for 6 days, and the exception exceeded the limit by less than 0.5 per cent. The normal location for 7 days was achieved by only 2 of the 4 blastocysts and 2 of the 6 models of similar size.

Another experiment was similarly scheduled and performed, but used 2-mm. models, appropriate in size to blastocysts 5 days *post coitum.* Only 1 of 5 models achieved the normal 7-day range. All 5 were within normal limits for either 5 or 6 days. The control blastocysts had an average diameter of 4.4

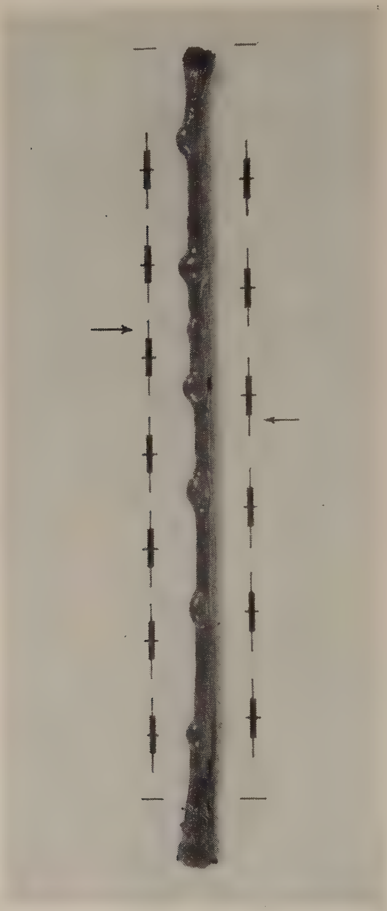

FIGURE 8. Blastocysts in a rabbit uterus at 7 days *post coitum* are approximately evenly spaced except for an abnormally small one (indicated by arrow on left). The adjacent lines indicate the most probable location, ½ standard deviation and 1 standard deviation for 7 (left) and 6 (right) blastocysts. All the blastocysts are within the limits (2 standard deviations) expected for either 6 or 7 normally located blastocysts, but the spacing is in best agreement with 6. Therefore, by measurement as well as by impression, the small blastocyst may be said to have been "ignored." The arrow on the right indicates the midpoint of the uterine horn. A general shift of a few per cent toward the cervix is evident. The ends of the uterine lumen (indicated by horizontal lines) were 206 mm. apart.

mm. (between the 6- and 7-day average). All were within normal 6-day limits; 6 of 7 were within the normal range for 7 days, and the exception was only 1.5 per cent beyond.

It is unfortunate, in these experiments, that models do not expand gradually as do blastocysts, and that it is impracticable to use models whose diameter is appropriate to 7 days *post coitum*, when the spacing is most regular. Nevertheless, it is quite safe to say that models are transported at the same rate and spaced with the same regularity as blastocysts of the same diameter, but lighter weight. Therefore, blastocyst diameter is the stimulus for spacing, and weight or nonphysical factors are not. The maternal factors, represented by the duration of gestation, are less critical than blastocyst diameter.

It may be asked fairly if this study of rabbit blastocyst transport can possibly have any relationship to blastocyst transport in primates, where there is only a single blastocyst, where that blastocyst does not expand greatly, and where the uterine muscle is vastly more massive and differently disposed. There are a number of illuminating similarities.

The rabbit blastocysts must have been propelled by spontaneous contractions originating near the uterotubal junction and near the cervix (to be exact, 4 per cent closer to the vagina than these points). No blastocysts ever lodged at the extreme ends of the uterine lumen; instead, the blastocysts nearest the ends were about as far from the end in question as from the nearest blastocyst. The end repulsion was illustrated dramatically, but in only one case, when a single blastocyst in a cornu $6\frac{3}{4}$ days *post coitum* was found at 45 per cent of the uterine length—essentially the uterine midpoint.

In two monkey uteri near to and at term (21 and 24 weeks), spontaneous contraction waves have been described as starting about 15 mm. from the uterotubal junction, passing forward and medially, and meeting at the midline. They often passed caudally, along the midline, and caused a shortening of the lower uterine segment. A circular contraction followed, which originated near the insertion of the round ligament. The contraction waves occasionally began unilaterally; some of these crossed the midline, whereas others did not. Reversed waves of contraction followed artificial mechanical stimulation of the lower part of the uterus (Ivy *et al.*, 1931). Although the preceding observations were made at the end of pregnancy rather than during the blastocyst transport period, it has been suggested that such opposed forces hold the blastocyst near the midline "as in an eddy" (Hartman, 1939). Thus macaques may resemble rabbits in having single blastocysts carried to and held near the middle of the uterus by opposed muscular forces from two sources, or "pacemakers," within the uterus.

The manner in which the muscles move the blastocysts also may be similar in the 2 species, if one may judge from the paradoxical rates at which contractions and blastocysts pass along the uterus. The contractions just described traversed the monkey uterus in about 3 to 8 seconds near term (Ivy *et al.*, 1931), when the distance from fundus to cervix is about 5 times as great as during blastocyst transport. The blastocyst, however, may require between 3 and 5 days to traverse only a portion of the uterus. If it is moved during all of that period, its average rate of travel is about 0.1 mm. per hour.

A similar paradox occurs in the rabbit. The propagated contractions observed during the time of blastocyst transport travel the length of the uterus about once a minute (Markee, 1944), whereas blastocysts take from 2 to 4 days and travel at an average rate of 1.2 mm. per hour the first 2 days and 0.6 mm. per hour the second 2 days. The paradox is present whether the cornu contains one or many blastocysts. Among 116 rabbit cornua in which blastocyst locations were determined, 3 had solitary blastocysts. Their locations in percentages of uterine length (near 200 mm.), were: tubal at 3 days, 6 pe cent at 4 days, and 45 per cent at $6\frac{3}{4}$ days *post coitum*. All were withi normal limits for their age. Thus, single blastocysts that were acted upo only by uterine end repulsion (and not by propulsion stimulated by neighborin blastocysts) were transported at 0.5 to 1.0 mm. per hour, or at nearly the rat (0.9 mm./hr.) that was average for multiple blastocysts between 3 and 7 days

There are several possible explanations for the disparity between the rates a which contraction waves and blastocysts travel, and they may apply equally t rabbit and monkey. It is conceivable that blastocyst transport may depen on muscle action that is so local or so deep that it has escaped detection. Thi cannot be evaluated at present. It seems likely that something may absor or cancel out the recognized muscular forces before they affect the blastocyst It also seems likely that something may impede blastocyst travel.

The impediment to blastocyst travel in the rabbit was thought (Böving, 1956

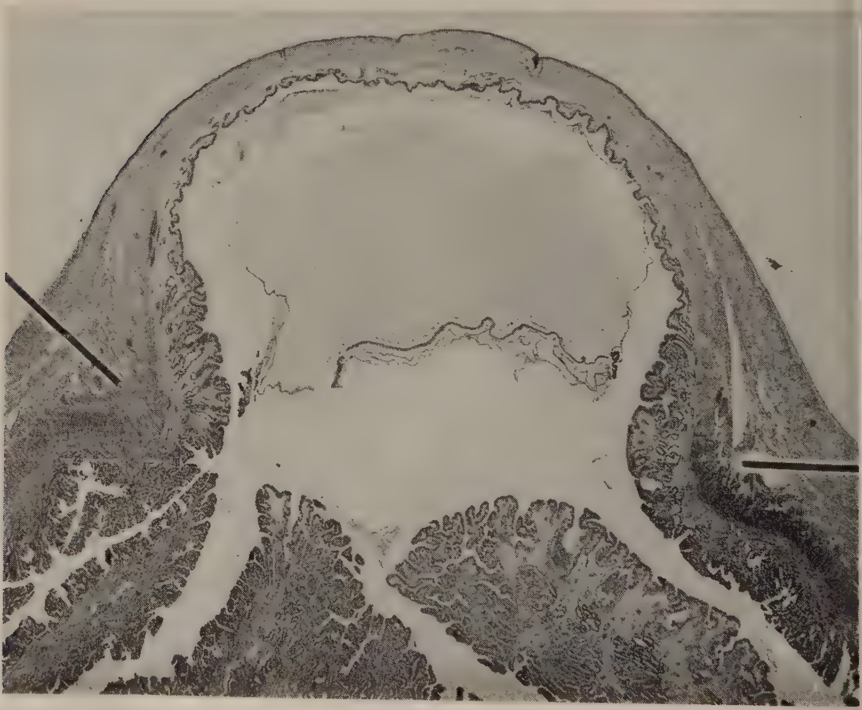

FIGURE 9. Shown in sagittal section, a rabbit blastocyst 8 days *post coitum* is lodged in a dome of attenuated uterus. Adjacent circular muscle forms an incomplete sphincter (at ends of pointers), which may hold the blastocyst in the dome. The noncellular membranes have been digested in the abembryonic (upper) hemisphere, where trophoblastic adhesion and penetration occur. They remain, with characteristically rolled-up edges, at the opposite part of the blastocyst. ×15.

to be related to size, because blastocysts travel faster when small than when large, and because glass models the size of blastocysts moved at the same rate as blastocysts, whereas stained sea urchin ova, which were much smaller, 0.074 mm. (Harvey, 1932), were conveyed much more rapidly (Markee, 1944). The matter must be reconsidered for three reasons. First, the amount of time spent in the uterus, presumably in transport, is remarkably similar for many mammals, regardless of extremes of blastocyst size (for example, rabbit) or myometrial massiveness (for example, primate). Second, it is known now that the rabbit uterus covers models as well as blastocysts with a mucous envelope that can be adhesive in some circumstances (Böving, 1957). A similar material was found in the uterine lumen of the monkey (884) killed during the period corresponding to blastocyst transport. Third, together with the sea urchin ova, referred to above, there had been injected into each rabbit cornu

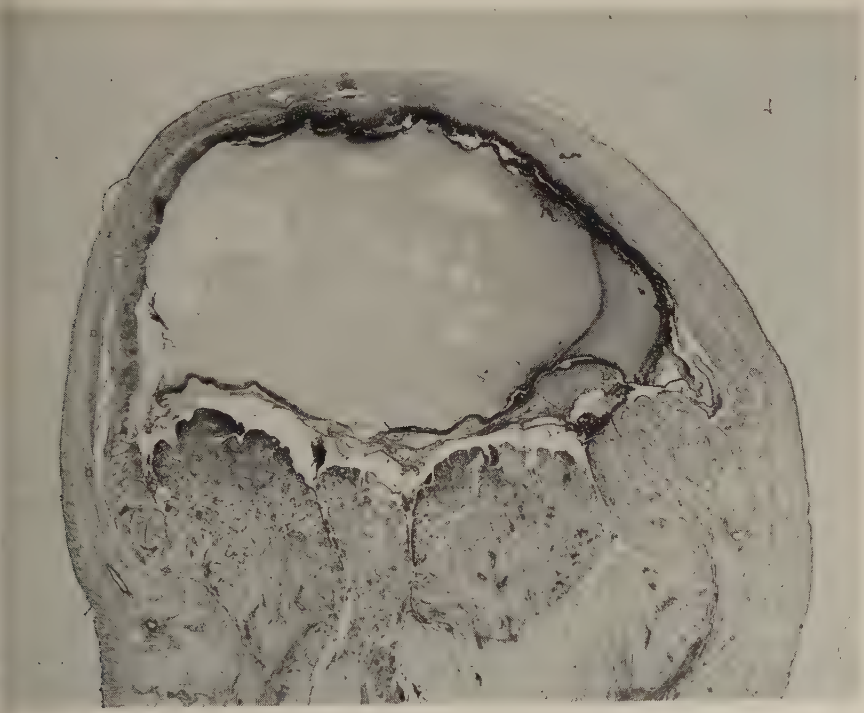

FIGURE 10. A transversely sectioned rabbit uterus 8 days *post coitum* has black precipitates antimesometrially and in the adjacent abembryonic part of the blastocyst. The precipitates follow perfusion with silver nitrate solution. Their absence in uterine tissues generally suggests that they are caused by something from the blastocyst. Their absence from the dead blastocyst (extreme right) suggests that the agent is a metabolic product rather than a stored substance. Other evidence (Böving, 1954) suggests that the precipitates indicate regions of high *p*H and carbon dioxide transfer from blastocyst to maternal circulation. The lumina of uterus and glands are almost absent in this perfused specimen, but are large in conventionally treated specimens, such as FIGURE 9. ×15.

0.1 ml. of normal saline solution. It is likely that even this small volume is an abnormally large amount of fluid. In pregnancy the uterine tube secretes an average of only 0.85 ml. per 24 hours (Bishop, 1956), and it is even uncertain that all of this enters the uterus. The uterus itself secretes an insignificant amount of fluid during the period of blastocyst transport. When opened both rabbit and monkey uteri are barely moist. The large lumina that uteri present histologically may be regarded for the most part as fixation artifact rather than spaces once filled with uterine fluid (compare FIGURES 9 and 10). It is suggested that the "eddy" in which primate ova were thought to be held (Hartman, 1939) may be a good figure of speech, but it may be improved by a reminder that the normal medium for blastocysts is both viscous and scant. The blastocyst *in utero* travels in mucus only a few microns thick.

The impediment of a viscous medium in the uterine lumen may not be the only reason for slow blastocyst travel. In effect, the rather mushy endometrium is an additional viscous fluid interposed between the blastocyst and the muscles that propel it. The softness or fluidity of the endometrium would tend to disperse equally any pressure exerted by the muscle, so that pressure ahead of a small blastocyst could not be much different from pressure behind At the same time, the endometrium could not flow far and carry the blastocyst with it, for it is anchored at its base. The endometrium and, presumably, the blastocyst would tend to return nearly to their previous locations at the end of each propulsive contraction. A more effective padding between muscle and blastocyst hardly can be imagined. It seems more curious that blastocysts are moved at all than that they are moved at only 1/1000 (rabbit) or 1/100,000 (monkey) the rate of the observed waves of contraction.

Grasp

It has been said that the attachment of the rabbit blastocyst to its implantation site may be said, in a way, to begin when the antimesometrial uterine wall yields to form a dome and the blastocyst becomes grasped in it (FIGURE 9). Since this muscular attachment is easily overwhelmed by distending or flushing the uterus, even in the rabbit, whose blastocyst is very large (Böving, 1952, 1954), it must be asked whether such a stage has not been missed in primates, whose small unimplanted blastocysts have almost necessarily been obtained by flushing rather than fixation *in situ.*

Adhesion by Noncellular Membranes

The shedding of the zona pellucida usually is said to precede blastocyst attachment to the uterine epithelium in mammals generally and in the primates specifically. The evidence for this is the recovery of free blastocysts without zonas. Zonas that were present in fresh specimens usually shrank and disintegrated locally during histological preparation, and it was recognized that one was ruptured during manipulation before fixation (Hertig *et al.*, 1954). Moreover, a normal macaque blastocyst (C-614) with a zona persisting until 8 days after ovulation is known (Streeter, 1938). It must therefore be conidered possible that the zona has been unnaturally lost from the free blastocysts rithout it. This caution is urged by several considerations. Human blasto-ysts still covered by the zona are sticky, as are blastocysts without (Hertig t *al.*, 1954). Rabbit blastocysts first become adherent to the uterus while till covered by a noncellular membrane whose thickness and physical and hemical properties are somewhat similar to the zona, although the origin is ubal and uterine rather than ovarian. In the rabbit, this membrane not only ermits adhesion, but is probably necessary to insure the consistent orientation f the blastocyst at the time of the initial adhesive attachment, since speci-nens adhering only by membranes were already properly oriented (Böving, 952 and 1954). It seems worthwhile to ask if the primate zona pellucida may ot also have a role in attachment rather than merely act as a presumably rotective encasement that must be shed before blastocyst adhesion can occur. n the other hand, uterine secretion, such as that which provides the rabbit lastocyst with its adhesive covering, perhaps should be studied as a possible nstrument of blastocyst attachment in primates.

An attempt was made to collect such uterine secretion from a monkey (884) y a method similar to the one used for rabbits. Paraffin pellets were placed in he uterus at the onset of the transport stage, day 16 of the cycle. At autopsy

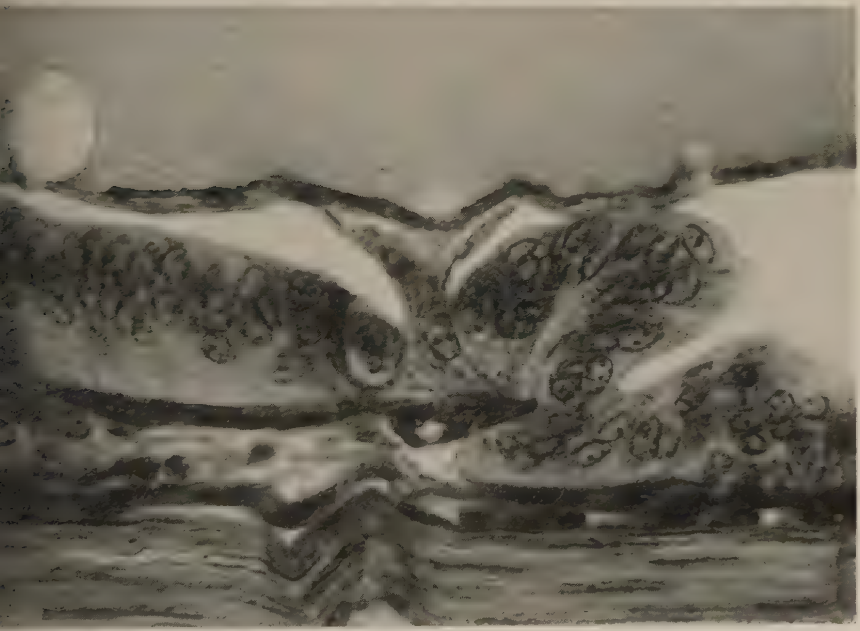

FIGURE 11. An unusually narrow rabbit trophoblast invasion has penetrated uterine epithe-um directly over a blood vessel. Intercellular membranes are obvious in the epithelium. pithelial degeneration is not widespread, but the cells immediately to the left of the invasion re pale and rounded into a clump that appears to dissociate from neighboring cells and sub-ucosa. ×600.

on day 19 the pellets were not recovered, but the uterine lumen and glands contained mucous secretion that resembled that of the rabbit and its "gloiolemma in amount, texture, and staining with azan and the Feulgen reaction. Thu during the transport stage, the monkey and rabbit uterine secretions are simila but it is still not known if and how the monkey uses the material for blastocys attachment. The role in attachment of human uterine secretion and stick blastocyst coverings should probably also be considered unknown rather tha unlikely.

Adhesion by Trophoblast

Freshly removed human (Hertig *et al.*, 1954) and rabbit blastocysts ar sticky after as well as before the noncellular envelopes have been removed Thus, blastocyst adhesion has two potential agents and, at least in the rabbit operates in two stages—first, the noncellular envelopes and, second, the trophoblast (Böving, 1954). Nevertheless, the underlying mechanism seems likel to be similar, for in both cases the adhesiveness is probably induced by high *p*H

General stickiness was induced artificially, when blastocysts were immerse in a solution with *p*H 9.5 to 10; this was reversed by acidification. A *p*H nea 9 was reached spontaneously in the abembryonic hemisphere of blastocyst

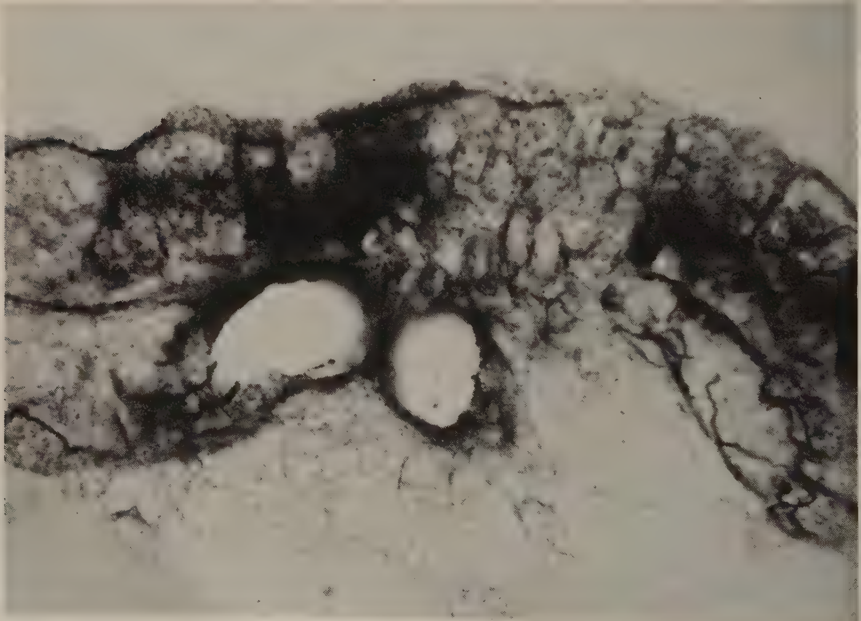

FIGURE 12. Silver nitrate perfusion at 7 days *post coitum*, when invasions are typically confined to epithelium with a vessel at its base, shows also that the pathway of chemical transfer from the uterine lumen to the maternal circulation is confined to the cells that have a vessel at their base. ×750.

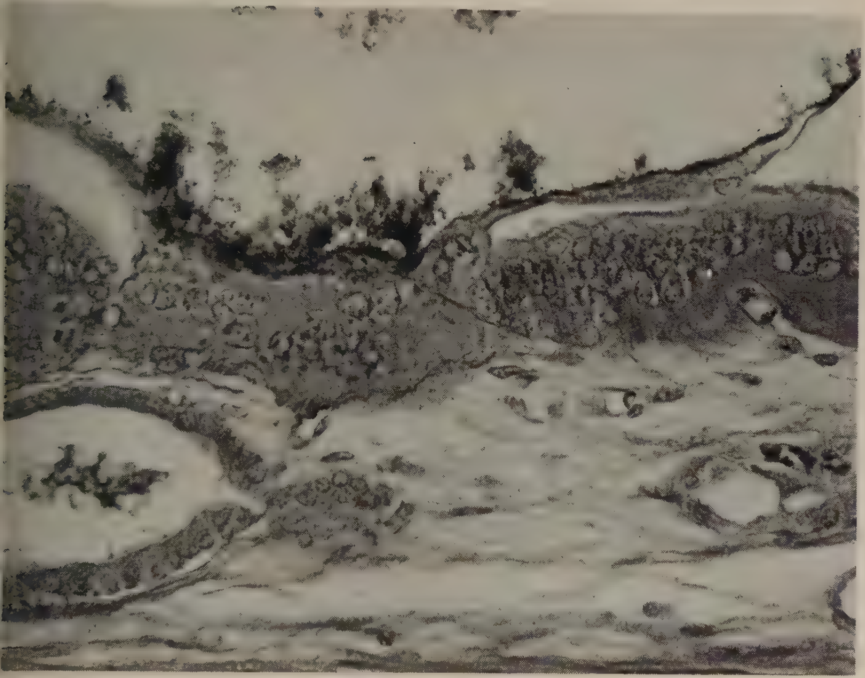

FIGURE 13. By 8 days *post coitum*, rabbit trophoblast invasions have spread widely in the lane of the epithelium, partly by pushing it aside and partly by destroying it. Intercellular embranes are not apparent in the epithelium. ×400.

emoved from the uterus and placed in unbuffered solutions containing indiator. The pH rise was attributed to carbon dioxide "blow off," because it was eversed by charging with carbon dioxide and because a precipitate was obained in barium hydroxide. Lead and silver precipitate at the same abemryonic location; it is here that the blastocyst adheres to the uterus, both efore and after the membranes are shed. That such a reaction may occur hile the blastocyst is still in the uterus is suggested by the antimesometrial recipitation of silver (FIGURE 10) when silver nitrate is applied by perfusion. f these precipitates can be considered to reveal alkalinity or carbonate, one has consistent array of *in vitro* and histological evidence for alkali-induced adesiveness both before and after the membranes are shed (Böving, 1954).

The early attachments of the blastocyst may occur in many places in the bembryonic hemisphere, and the alkaline reaction, on which it is thought to epend, may be elicited widely when the blastocyst is removed from the uterus, ut the separate attachments (FIGURE 11) are themselves initially quite small 10 to 20 μ across). Thus, in the uterus at 7 days *post coitum* all the requirements for attachment are met at only restricted points in the general abembrynic or antimesometrial region. If the discrete attachments are regarded as

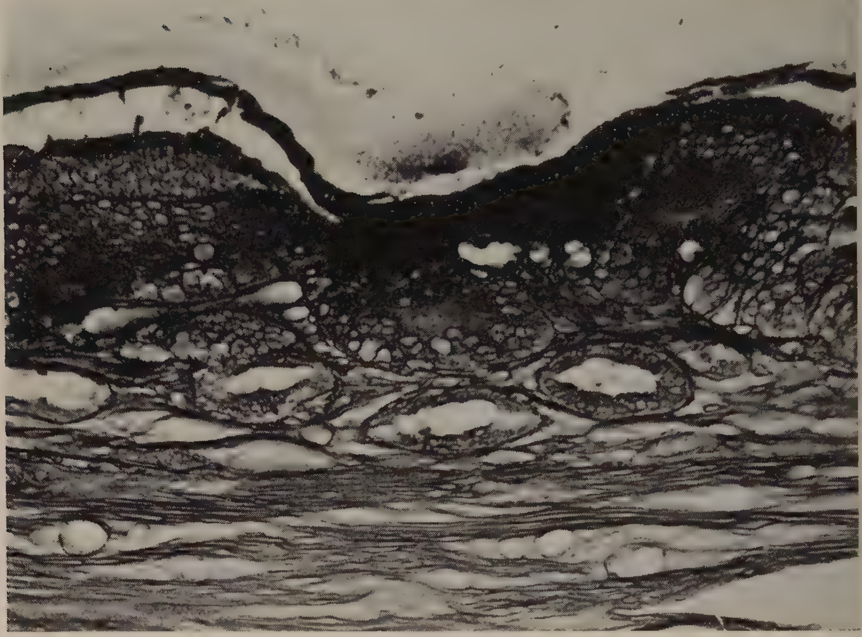

FIGURE 14. At 8 days *post coitum*, silver nitrate perfusion shows that the chemical pathway, like the invasions, has spread. Intercellular membranes have disappeared from the epithelium near the invasion, but a few remain at the extreme right. ×200.

histological pointers, one may discern that attachment is privileged where the uterine epithelium has a capillary at its base. Apparently the potential for the alkaline reaction is widespread in the abembryonic part of the blastocyst, but it is elicited preferentially where the uterine epithelium has a vessel at its base. This is understandable if the epithelium with a vessel at its base is a preferred and narrowly restricted site for removal of carbon dioxide from the blastocyst and, thus, for induction of high *p*H and accompanying adhesiveness. That such is the case is suggested (FIGURE 12) by the preferential precipitation of silver in the epithelial cells that rest on capillaries perfused with silver nitrate solution (Böving, 1954).

At 7 days *post coitum*, the narrow channel for chemical exchange between blastocyst and maternal circulation, which the silver perfusion defines (FIGURE 12), corresponds in location and width to the narrow trophoblast attachments to the epithelium (FIGURE 11). At 8 days *post coitum*, trophoblastic attachments are much wider (FIGURE 13), and the silver precipitates are so diffuse as to suggest that chemical transfer is no longer channeled (FIGURE 14). Since the intercellular membranes of the uterine epithelium are present at 7 days and disappear at 8 days *post coitum*, they may be held responsible for channeling the chemical exchange and establishing the precise "aim" of the trophoblast for vessels at 7 days. The subsequent spread of trophoblastic attachments may be attributed to their disappearance (Böving, 1955).

Invasion

The selective adhesion to epithelium with a vessel beneath explains only the "aim" of the subsequent trophoblastic invasion. It does not explain the mechanism by which the epithelium is penetrated. The anatomy of penetration in the macaque has been described in painstaking detail (Heuser and Streeter, 1941). Briefly, these investigators found penetration by, or at least accompanied by, epithelial destruction. An opposite view is that histological appearance does not suggest that trophoblast penetrates epithelium by digesting it (Mossman, 1937).

The difference of interpretation appears to be explainable. In the rabbit, well-established penetrations of a specimen 8 days *post coitum* show both a pushing aside of epithelium and some very local damage to and removal of epithelial cells. Blood vessels attached to the base of uterine epithelium were considered markers. The average distance between 685 of them was $97.3 \pm 2.6\ \mu$. The 110 of them that were at trophoblastic penetrations, or on either side of penetrations, were separated by an average of $113.9 \pm 7.1\ \mu$. Thus, vessels at invasions were 17 per cent further apart than vessels generally, and the difference was significant ($P < 0.02$ by "t" test). Since epithelium and vessels were attached, this implies that the epithelium was pushed aside an average of about $17\ \mu$ at each trophoblastic penetration.

The average epithelial destruction at these sites of penetration was measured by the difference between the above epithelial displacement and the average distance ($71.2 \pm 1.4\ \mu$) over which trophoblast had replaced epithelium. The amount was about $55\ \mu$. Since one half of this is on one side of a penetration and one half on the other, and since epithelial cells are 5 to $10\ \mu$ across, the average trophoblastic penetration in the specimen studied must have destroyed and substituted itself for a zone of adjacent epithelium 3 to 6 cells wide.

The preceding analysis does not show whether the displacement and destruction it distinguished occur simultaneously, alternatively, or in turn, or overlap. This is so because, even in a single specimen and even with restriction of sampling to invasions that have completely penetrated the epithelium, the sample includes many stages of invasion. A preliminary application of the above procedure to a younger specimen did not answer conclusively the question of sequence. For the moment, all that can be offered is my impression that, at very small and presumably beginning attachments, the uterine epithelium of the rabbit yields mechanically before there is histological evidence of injury (FIGURE 15), whereas the reverse sequence occurs in the macaque (Heuser and Streeter, 1941), very obviously at the secondary site of placentation and more subtly at the primary site (Wislocki and Streeter, 1938).

The fate of the epithelial cells replaced by the trophoblast is obscure. There is little proof that they are absorbed by the trophoblast (Mossman, 1937). In the rabbit, the epithelium adjoining trophoblastic penetrations looks as if it is being neither digested nor ingested. Necrosis or epithelial debris are not

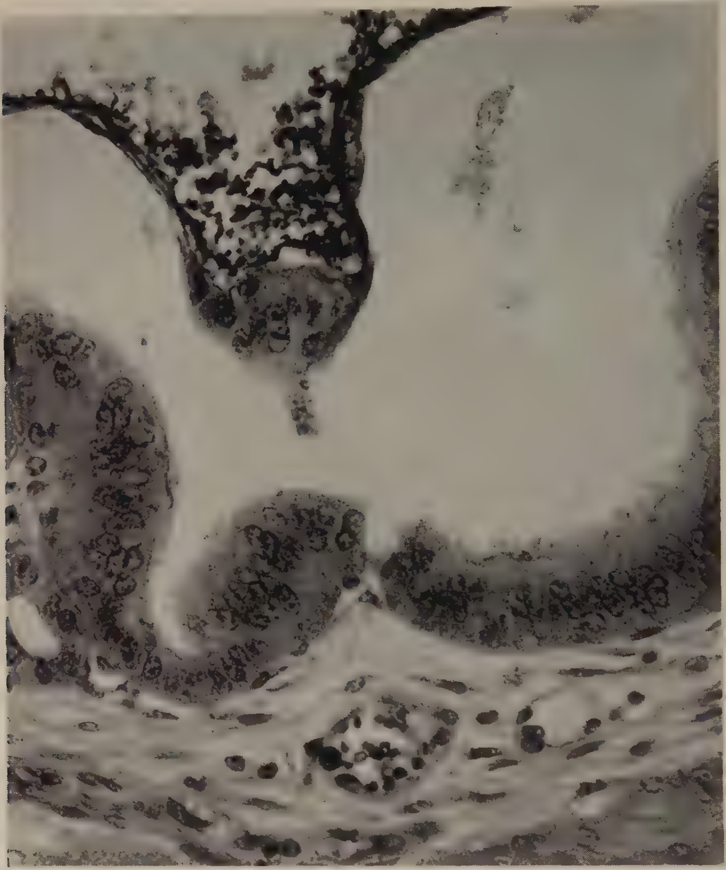

FIGURE 15. This revealing artifact shows that trophoblast adhesion to an epithelial cell may be stronger than epithelial cohesion (although this is not usually the case). It suggests that trophoblast penetration is preceded by dissociation of the epithelial cells that lie between the trophoblast and the vessel, possibly by the same chemical mechanism to which adhesion is attributed. ×400.

consistently present, but they do occur. Perhaps they are missed, since only a few cells are removed, and even these few may be destroyed one at a time. Alternatively, penetrations are occasionally adjoined by pale cells, rounded either singly or into a clump, as if they had become detached from the submucosa and the neighboring intact epithelium (FIGURE 11). Rarely, somewhat similar cells or their nuclei are seen in the uterine lumen. Perhaps some of the epithelial cells replaced by the trophoblast are not digested or ingested, but merely loosened and shed into the uterine lumen.

A possible mechanism for trophoblastic penetration of rabbit uterine epithelium was suggested by an incidentally observed artifact (FIGURE 15). Following trophoblast adhesion to epithelium, but before penetration, the epithelial cell that adhered to trophoblast lost its ability to cohere with its neighbors. Such local epithelial dissociation could be presumed to "clear the path" for trophoblast penetration. Moreover, in view of its association with both a vessel and a trophoblast adhesion, the inferred high pH in this area and the known dissociative effect of high pH (Holtfreter, 1944), the yielding of the epithelium may be presumed to depend on the same chemical mechanism that caused the localized trophoblast adhesion. It is singularly appropriate to this view that, during penetration, the invading tissue is syncytial and, therefore, not susceptible to dissociation, and that antimesometrial invasion becomes arrested at about the time that uterine epithelium loses its intercellular membranes and itself becomes syncytial. Moreover, it explains both the epithelial displacement and replacement as consequences of a single mechanism—epithelial dissociation. This seems a simpler hypothesis than the alternatives that come to mind. Nevertheless, it is only a hypothesis, as yet without experimental confirmation.

Passage Through the Epithelium and Shedding of the Zona Pellucida

Even if one were sure that epithelial dissociation cleared a path for trophoblast invasion, it would remain necessary to explain how the trophoblast is pushed between the dissociated cells. The process is probably quite rapid in the rabbit, since partial penetrations are rarely seen. Guinea pig blastocysts cultured *in vitro* exhibited local penetration of the zona pellucida by numerous fingerlike projections of the attachment cone (Blandau and Rumery, 1957). The zona covering the rest of the blastocyst remained unchanged. The projections had ameboid and undulating motion. Such motion seems adequate to explain rapid trophoblast penetration through loosened epithelium.

In the rabbit, as in the guinea pig, the encasing noncellular membranes are perforated selectively in the abembryonic hemisphere where they overlie trophoblast knobs, the part of the trophoblast destined to invade them. Soon the entire abembryonic part of the membranes degenerates, whereas the membranes at the embryonic hemisphere remain relatively intact (FIGURE 9) until they are shed (Böving, 1954, 1957). A comparable stage of *Citellus tridecemlineatus* is illustrated by Mossman's Figure 13 (Mossman, 1937). There s no exactly comparable specimen of the monkey, but FIGURE 2 shows that its zona is present at 8 days, nearly the time of implantation, and that it is then more degenerate at the embryonic and abembryonic poles than equatorially. The areas of degeneration overlie the regions of trophoblast that are destined to invade, just as in other species.

It is not certain that the same process occurs in the human. A human blastocyst (8794) of 58 cells has a corresponding deficiency of the zona pellucida at the embryonic pole, but the blastocyst is so young, 3 days, that it seems unikely to be related to implantation. Moreover, a similar defect has occurred already in a 2-cell ovum (8698). The lack of a zona around the oldest (8663) ree blastocyst (Hertig *et al.*, 1954) casts further doubt although, as has been pointed out, the loss of the zona may be an artifact.

Even if the human zona pellucida were perforated locally by trophoblast near the time of implantation, as in the other species, it is not obvious that this would explain the massive passage of an entire blastocyst through the epithelium and into the submucosa. However, it might help. The turgidity of the human blastocyst before implantation and the flaccidity immediately afterward suggest that the internal pressure may have something to do with the passage. However, such an internal pressure, exerted equally in all directions, could only expand the blastocyst. It could not push the blastocyst through the epithelium unless expansion of the blastocyst were confined and thus directed. If the human zona pellucida, like that of the guinea pig, rabbit, and perhaps of the monkey, persisted everywhere except in the region of invasion, it could conceivably provide the required confinement for the expansion and cause extrusion of the blastocyst out of the zona pellucida and through the epithelium.

This interpretation may be challenged by the observation that both macaque and human blastocysts are turgid before and flaccid after implantation, yet the human implants below the epithelium and the macaque does not. In answer, one must admit that uncertainties are serious but, insofar as one can judge, the human zona destruction is unipolar, whereas the macaque's is bipolar. Under the above hypothesis, this could favor the human blastocyst's extrusion through the epithelium and perhaps even leave some intact zona to block invasion of the opposite wall of the uterus. The reluctance to conclude that human zona destruction is unipolar at the time of implantation comes chiefly from the fact that the specimens in which destruction has been seen are so young. If one actually sees early destruction and not artifact, it is consistent with and perhaps contributive to the 3-day acceleration of human implantation relative to the macaque.

Conjecture such as the above is, of course, reprehensible if there is hope of a direct answer, but this seems unlikely. It is difficult to obtain any specimens at all, either human or chimpanzee, at so early a stage of pregnancy. Moreover, if the instant of passage were as brief as rabbit trophoblast penetration, it would probably be missed. Finally, it is impracticable to prepare sections without opening or flushing the uterus, and this must be expected to disturb any delicate balance of forces.

Submucosal Invasion

Trophoblast invasion in the submucosa has been studied in the human (Krafka, 1941) and in the macaque (Wislocki and Streeter, 1938). Among various opinions mentioned by Krafka, it has been held that trophoblast grows and displaces stroma but does not injure it, that trophoblast grows around vessels but does not erode them, that trophoblast grows around vessels and glands and erodes vessels but not glands, and that trophoblast invades both vessels and glands. Clearly, there is no consensus.

Krafka noted that uterine vessels might break down even where there was no trophoblast in juxtaposition. This was thought to show that trophoblast is not necessarily the cause of uterine vessel erosion, although the two are usually associated (Krafka, 1941). Since mouse trophoblast placed in the eye causes hemorrhage (Grobstein, 1950), Krafka's observation may serve better to show that the trophoblast produces a vessel-destroying substance that diffuses into the submucosa. No spontaneous vessel erosion or leakage is evident in Bartelmez' photographs of nonpregnant macaque uteri injected at stages of the cycle corresponding to the time of implantation (Bartelmez, 1957).

Near the time of implantation and under hormonal control there is reduced capillary flow, which causes submucosal edema, most pronounced near the epithelium. This is thought to favor trophoblast invasion (Bartelmez, 1957). Also the decidual reaction (large, pale cells) of the submucosa has been regarded as a maternal promoter of invasion. An opposite role, maternal protection against invasion, is favored by Krafka (1941), who points out that a similar reaction occurs at sites of ectopic implantation.

The regeneration of the uterine epithelium over the path where the ovum has passed poses a problem, for it occurs while trophoblast is next to it and while blood vessels not so far away are being engulfed and eroded by trophoblast (FIGURE 5). If destructiveness is an intrinsic and general property of trophoblast, then its agent must have tissue specificities that vary with time or environment. If, on the other hand, the regional differences in effect depend on different anatomical and physiological types of trophoblast in the different regions, then one must explain how each type develops at the proper time and place. In either case, the trophoblastic and endometrial behavior are almost certainly regulated by an interplay of embryonic and maternal factors, but there is no comprehensive explanation.

Summary

(1) Rabbit blastocyst attachment to the uterus depends on three principal mechanisms: muscle, adhesion, and invasion. These operate in sequence but with some overlap.

(2) Uterine muscle is the agent that transports blastocysts from the uterotubal junction to their lodging place and holds them there. First, from 3 to 5 days *post coitum*, blastocysts are scattered randomly along the length of the uterine horn. Second, from 5 to 7 days *post coitum*, blastocysts expand and thereby cause the uterus to space them at equal distances from each other or an end of the uterine horn. Both blastocyst expansion and the appropriate uterine reaction to it depend on progesterone and probably an absence of estrogen. Third, at 7 days *post coitum*, blastocysts have expanded so much that they can no longer be propelled by the uterus. The antimesometrial muscle loses tone over each blastocyst, and the blastocyst lodges in the resulting dome. Adjacent circular muscle becomes disposed in bands (like an incomplete sphincter) that grasp the blastocyst and hold it in the dome. This grasp may be considered a muscular phase of attachment; it insures that subsequent attachment will be antimesometrial.

(3) Adhesion of the blastocyst to the uterus begins abembryonically with noncellular membranes that surround the blastocyst. This insures that subsequent attachment will be abembryonic. The adhesion is probably alkali induced. Trophoblast adhesion to the uterus follows, when the noncellular

membranes disintegrate locally near trophoblast knobs destined to invade. Membrane disintegration soon spreads over the whole abembryonic hemisphere. Trophoblast adhesion is privileged where the uterine epithelium has a capillary at its base.

(4) Invasion, like adhesion, is privileged where the epithelium has a capillary at its base, and it is believed to be induced by local high pH attending a transfer of carbon dioxide or related substance to the maternal circulation. In a specimen 7 days *post coitum*, the "aim" for vessels was extremely precise. In a specimen 8 days *post coitum*, adhesions and penetrations were much wider, and the "aim" was less precise. The difference is explained by the disappearance of intercellular membranes from the uterine epithelium and conversion of a narrow pathway of chemical exchange between blastocyst and capillary into a diffuse one. Trophoblast penetrates uterine epithelium by both displacement and destruction. A single mechanism, cell dissociation by high pH, conceivably may be responsible for both aspects of invasion.

(5) The events of human implantation can be outlined only broadly, because there are no specimens of implantation in the strict sense of blastocyst passage from the uterine lumen to the submucosa. A comparative study suggests some of the mechanisms and events that remain unknown.

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VASCULAR ADAPTATIONS OF THE UTERUS TO PREGNANCY

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It may be well, at the outset, to dispel a misapprehension implied in the title of this discussion. Our orientation toward the cyclic changes in uterine vasculature is quite false if we look upon pregnancy as an isolated event for which special and extraordinary preparations must be made. This attitude, which underlies more of our thinking than we are perhaps aware, stems from the preoccupation of both clinicians and investigators in the last few decades with the phenomena of the menstrual cycle. Analyses of "the cause of menstruation," studies of "the mechanism of menstruation" have tended to set the menstrual cycle apart as a separate entity in the over-all reproductive pattern. By implication, pregnancy is also separate and different. That the two processes dovetail, that one is a "preparation" for the other, has not been doubted, but a sort of anatomical and physiological gear shifting has been assumed when pregnancy supervenes in the orderly succession of menstrual cycles. The accumulating data of recent investigations and their thoughtful interpretation in the framework of reproductive physiology as a whole indicate that no such dichotomy exists in fact or is justified in concept, even as a matter of convenience in description.

The classic experimental studies of Daron,[1,2] of Markee,[3] and of Bartelmez,[4,5,6] made twenty years ago on the nonpregnant endometrium in the rhesus monkey and man, laid the foundations of our understanding of uterine vascular physiology. Subsequent studies of placental vasculature at the laboratory of the Carnegie Institution of Washington and elsewhere, to be summarized in the present report, have approached the same basic problem from the other end. In two recent papers of high significance, Bartelmez[7,8] has interpreted the data obtained from these angles of approach and has brought clearly into focus the fact that specific adaptations for pregnancy commence on day 1 of the menstrual cycle and form a smooth, unbroken, purposeful sequence thereafter.

The dual arterial supply to the primate endometrium and the sensitivity to hormonal stimulation of the Type I (spiral or coiled) arteries that supply its superficial one third are the pivotal factors in the cyclical behavior of the endometrium. This sensitivity is manifested during the follicular phase of the cycle by rhythmic dilatation and constriction of the superficial capillary bed supplied by the spiral arteries and in the progravid phase by rapid growth of these arteries when the blood level of corpus luteum hormone is high and by vasoconstriction when the level is reduced. The rapid growth, outstripping that of the endometrial stroma, leads to increased coiling which, in turn, retards circulation in the capillary bed with production of edema. The constriction of the arteries creates stasis and consequent ischemia and extravasation of blood. These latter phenomena occur in all cycles at about days 24 to 28, when the activity of the corpus luteum is commencing to wane. The causative vasoconstriction

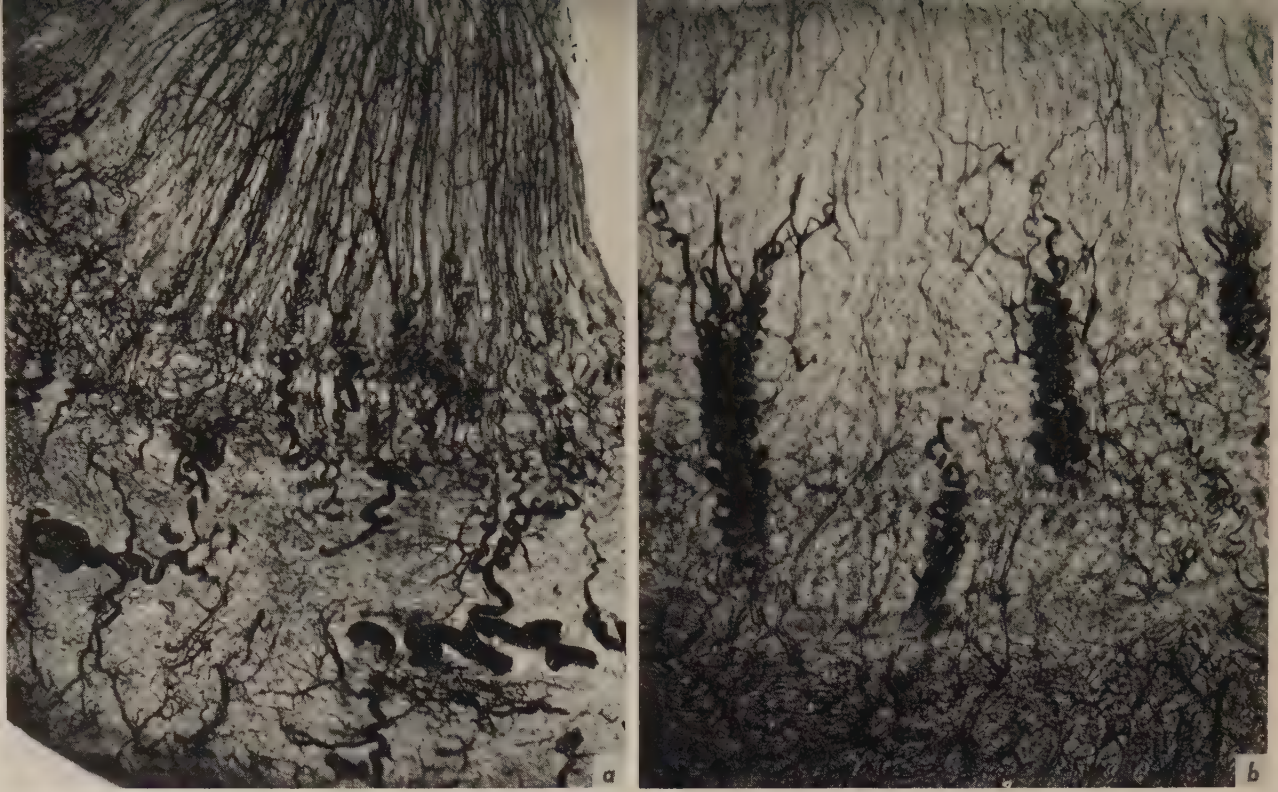


FIGURE 1. (*a*) A cross section of the uterine wall of a monkey on day 12 of the menstrual cycle. A mature follicle was demonstrated in the ovary. A spiral arteriole displays minimal coiling in the lower third of the endometrium. Its straight distal end reaches half way to the surface epithelium. India ink and starch injection. B 338. ×15. (*b*) A cross section of the uterine wall of a monkey on day 17 of the menstrual cycle. A 6-day-old corpus luteum was demonstrated in the ovary. The abundant coils of several arterioles are seen in the middle third of the endometrium. The straight distal ends reach three fourths of the way to the surface epithelium. India ink and starch injection. B 309. ×15. FIGURES 1*a* and 1*b* reproduced from Ramsey.[22]

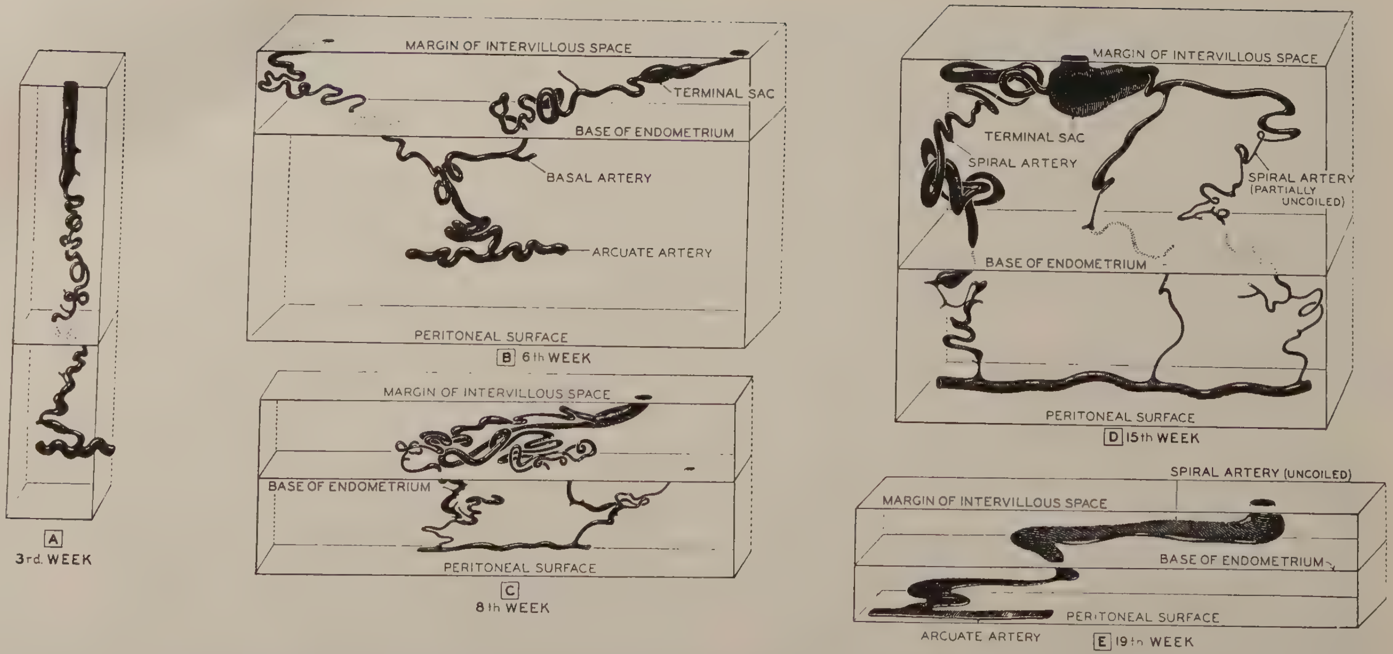


FIGURE 2. Diagrammatic representations of the course and configuration of the spiral arteries of the maternal placenta of the monkey at various stages of pregnancy, based upon three-dimensional reconstructions of serial sections.

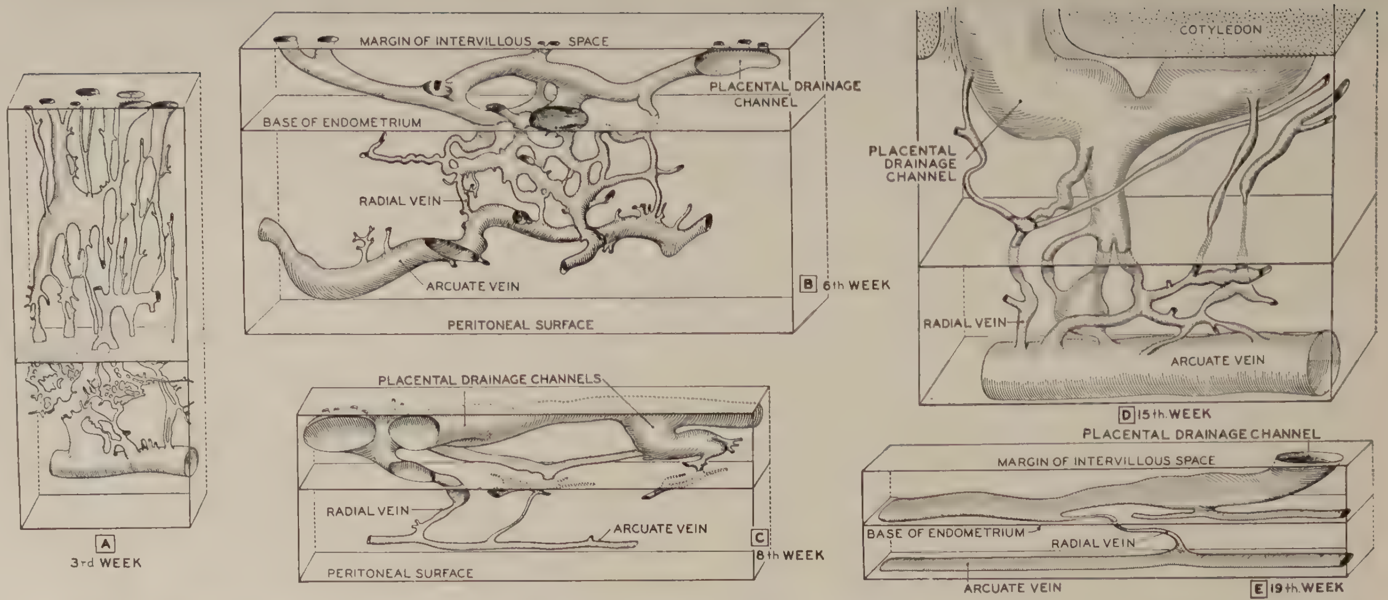


FIGURE 3. Diagrammatic representations of the course and configuration of the veins of the maternal placenta of the monkey at various stages of pregnancy, based upon three-dimensional reconstructions of serial sections.

is confined to the arteries supplying the paramedian region of the corpus and fundus, that is, the potential implantation sites. The sensitivity threshold of these arteries is lower than that of the rest.

If no ovum implants in a given cycle and the action of the corpus luteum is terminated, the vasoconstriction spreads to all of the spiral arteries, resulting in menstrual bleeding and slough, and arterial growth ceases. If implantation does occur, however, the corpus luteum hormone level rises again and arterial growth resumes, after the brief period of local disruption and extravasation the so-called "implantation bleeding" which is usually inconspicuous in the human, but overt in the macaque. The damaged endometrial tissue serves as pabulum for nourishment of the ovum before placental circulation is established and the edema evoked by retarded capillary circulation facilitates trophoblastic invasion. The slowing of the circulation itself helps prevent dislodging of the implanting ovum by maternal blood as the trophoblast opens up the superficial capillaries.[6–8]

Comparison of the configuration of the arteries illustrated in FIGURES 1*a* and 1*b*, taken from days 12 and 17 of the cycle, with those represented in FIGURE 2 shows clearly that the transition through the phases of the menstrual cycle and on into pregnancy is a smooth and uninterrupted one. The form of the first spiral artery tapped by the invading trophoblast, just before day 19, is altered quantitatively but not qualitatively from that of the preimplantation, progestational artery. Growth, initiated many days earlier, has produced increased coiling and to it are added, as pregnancy progresses, back and forth and lateral looping, so that the elongating artery can accommodate itself to the decrease in diameter of the endometrium occasioned by pressure of the overlying conceptus.[9]

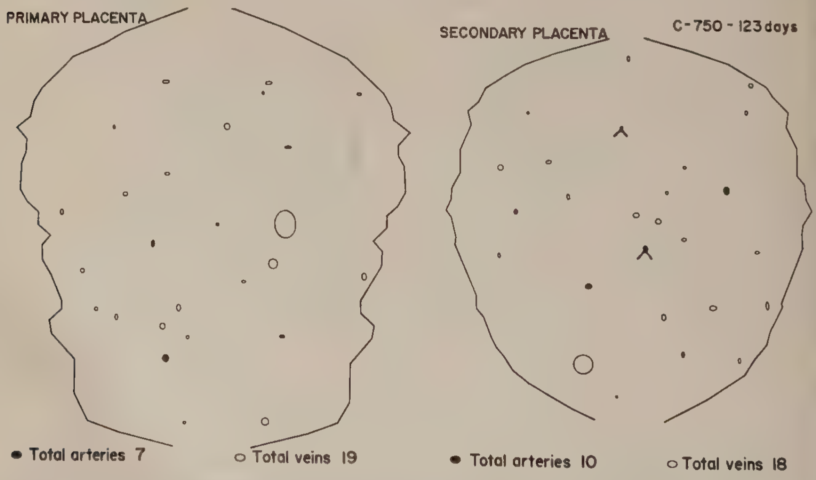


FIGURE 4. Graphic diagrams of the two placental discs of a representative, mature monkey pregnancy on which are plotted the orifices between the intervillous space and maternal arteries and veins. In the secondary placenta are seen two instances in which two placental arteries enter the IVS through a single orifice. Reproduced from Ramsey.[25]

At mid-term “conversion” occurs, so named by Reynolds,[10] who showed, in
arious species including the rhesus monkey and man, that at this time increase
n the size of the uterus abruptly ceases to be accomplished by growth of com-
onent parts and is continued by mechanical stretching alone. At conversion the
oils of the spiral arteries are paid out, and thereafter until term the course of
he vessels is a gently undulating one.

With this change and the concomitant passive dilatation and stretching of the
aternal veins connecting with the intervillous space (FIGURE 3), we may look
pon the pattern of maternal placental vasculature as matured and settled.[9, 11]

It now becomes necessary to inquire how many connections there are between
he placenta and the maternal circulation and what their distribution is. If,
r example, there were but one artery and one vein, the clinical significance of
heir occlusion would be drastic; or, if the connections were localized to a single
egion of the placenta, pathological destruction of that area would be of fatal
onsequence. What, in other words, is the margin of safety in the placenta in
rms of the number of maternal vascular connections with the intervillous
ace and of the distribution of the orifices of communication?

Only Spanner[12] has made an actual count of placental arteries. In a single
uman placenta at 7½ months, he found 94 spiral arteries connecting with the
lacenta. In the human, however, individual arteries tend to split into a number
f terminal mouthpieces that enter the intervillous space separately, so that
panner's total count of actual openings into the space was 488. Boyd[13] com-
uted total orifices in 6 human placentas, basing his estimates upon extension
f sample counts to the total placental area. In 3 placentas at 4 months, the
nge was 102 to 156, and in 3 at term, 180 to 320.

In the monkey arterial stems do not enter the intervillous space by multiple
outhpieces. On the contrary, adjacent arteries occasionally join close to the
lacental base and enter via a common dilated terminal sac (see *C* and *D* in
IGURE 2). To determine the number and distribution of the vascular connec-
ons in the monkey, both primary and secondary placentas from a group of
nimals in the third trimester of pregnancy were embedded *in toto* and serially
ctioned. Arterial stems and openings and also venous drainage orifices were
unted. In the graphs reproduced in FIGURE 4 the location of these orifices in
representative case has been plotted. The total number of connections (17
teries and 37 veins) is somewhat smaller than might have been expected upon
e basis of comparative placental areas in human and monkey; somewhat
naller also than might be expected if one computes the number of spiral arter-
s in an area of the progravid endometrium equivalent to the placental area
artelmez[8] found an average distance of 1.4 mm. between arteries in the late
ogravid phase). The latter discrepancy rests upon the fact that a large pro-
rtion of placental growth is interstitial, so that attachment is actually made to
surface of the endometrium smaller than the total area of the base of the
acenta. The spotty distribution of the orifices of vascular connection, as
en in the graphs in FIGURE 4, shows that estimates based on sample areas are
cessarily misleading. A more important feature of the distribution, however,
its general uniformity with both arterial and venous openings appearing in all

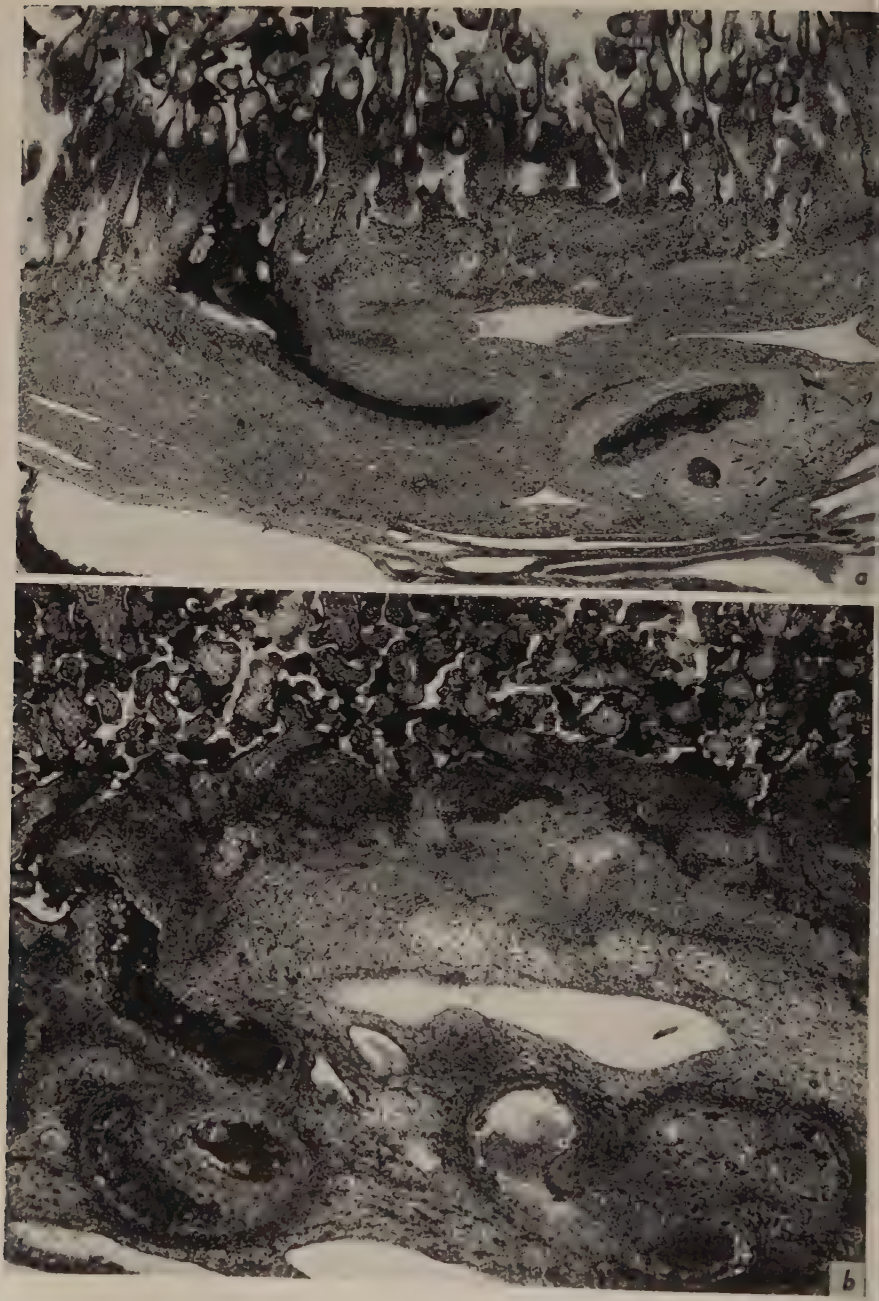


Figure 5

portions of the placental base, central and marginal alike, and the two sorts of openings indiscriminately mixed.

Although the above findings certify the existence of an adequate margin of safety in placental vasculature, a physiological dilemma is presented by the haphazard distribution of the orifices of afferent and efferent channels: namely, why does not entering maternal blood short-cut at once to the nearest adjacent venous orifice and leave the placenta without having circulated throughout the intervillous space? This dilemma has been a matter of concern to investigators for a long time and has prompted the elaboration of various theories of placental circulation. The evidence of recent studies has undermined some of the previously accepted theories that prove to have been based upon inadequate or imperfectly understood anatomical findings; at the same time, however, the new evidence has led to the formulation of a physiological concept of placental circulation.

Bumm's[14] theory, promulgated in the 1890s, envisaged entry of arterial blood into the intervillous space exclusively through orifices located high on the placental septa whence, he believed, it flowed back toward basally located venous outlets, bathing the villi on its way. Spanner's[12] theory, which replaced this forty years later, accepted basal arterial entries, but denied venous outlets anywhere except at the margin of the placenta. Spanner viewed circulation in the intervillous space as an "overflow" mechanism with arterial blood entering through the basal orifices and rising between fluid-tight maternal septa up to the subchorial lake, thence, by lateral diffusion, progressing to the marginal sinus to be drained into the maternal venous channels.

Recent experimental work in several laboratories has provided evidence counter to that upon which these earlier classic theories were based (see Bøe,[15, 16] Boyd,[13] Browne,[17, 18] Hamilton and Boyd,[19] Kladetsky-Haubrich,[20] Ramsey,[9, 11, 21–25] and Stieve[26, 27]). The investigations carried out at the Carnegie Institution consisted of injection of a large series of pregnant monkeys *in vivo* and of more than 30 postoperative human uteri with placenta *in situ*. The monkey specimens were prepared by one of three intravital methods: (1) intra-aortic injection of India ink;[9, 11] (2) femoral vein injection of mercuric sulfide;[28] and (3) autoinjection[25] achieved by bathing the *in situ* uterus in copious amounts of Bouin's solution for 3 to 7 min. before clamping the uterine ligaments with their contained vessels. In the interval of persisting circulation, the interaction between the Bouin's fluid and the blood so changed the color of the latter that autoinjection was obtained. The human material, consisting of operative or, in 2 instances, post-mortem cases was postvitally perfused via the uterine arteries with a 1 per cent aqueous solution of sodium nitrite, with 0.1 per cent histamine,

FIGURE 5. (*a*) A portion of the base of a monkey placenta; (*b*) a similar section from a human placenta. In each, a maternal spiral artery is seen at its point of entry into the intervillous space at the left of the photograph with proximal coils of the same vessel at the right. It is to be noted that the entries are basally located and are not associated with maternal septa. Note also the similarity of pattern in monkey and human. Monkey C 658. Thirty-eighth day of pregnancy. Slide B 35. Hematoxylin and eosin. ×30. Human 8593. Twenty-second week of pregnancy. Slide A 156. Hematoxylin and eosin. ×30. Reproduced from Ramsey.[22]

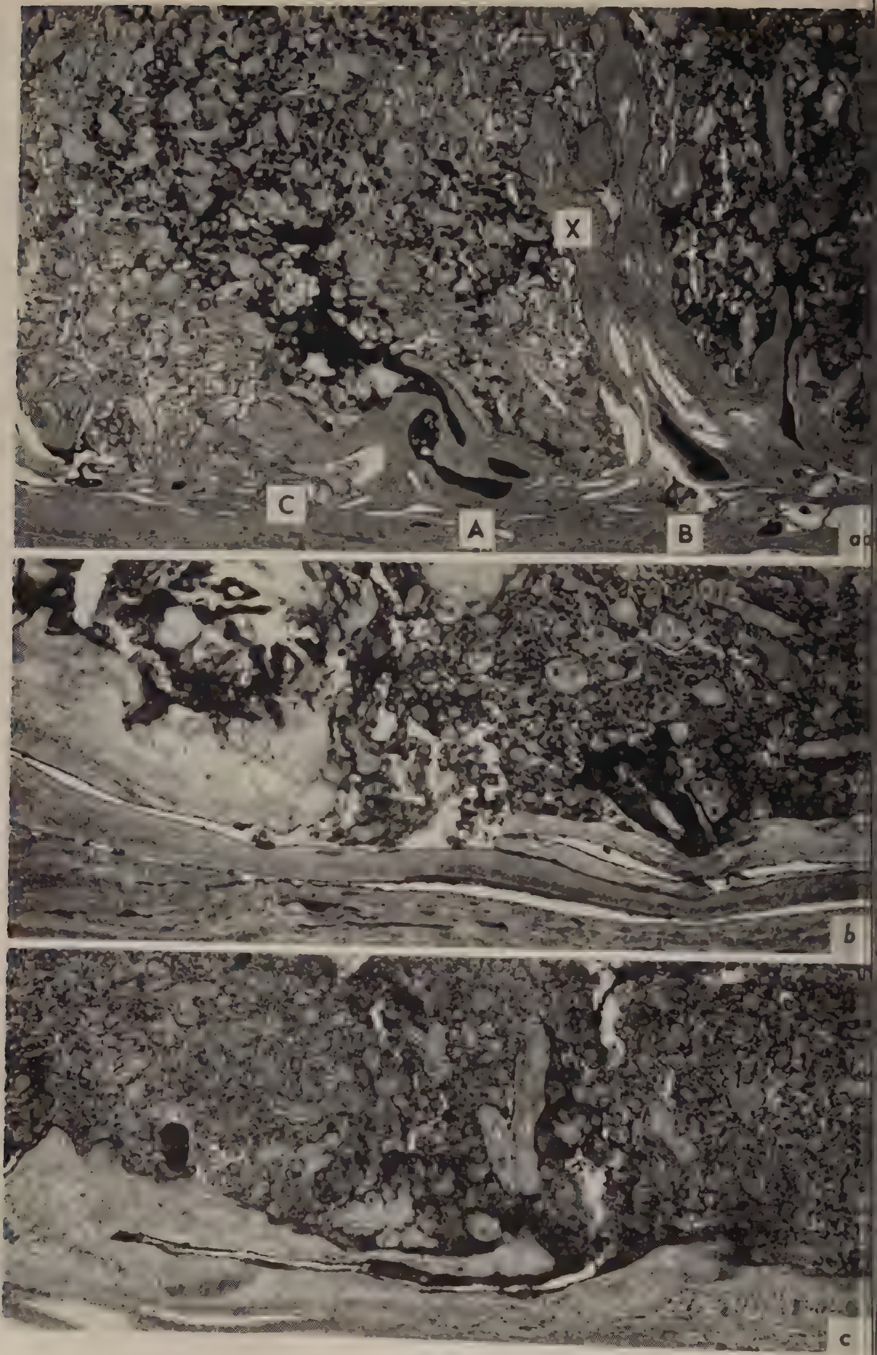


FIGURE 6

ollowed by India ink introduced at pressures just below that of maternal arterial lood.[23] As illustrated in FIGURES 4 through 7, the evidence of these injections lemonstrates that both arterial and venous orifices are basally located and that enous drainage occurs centrally as well as marginally.

A large and carefully studied body of human material reported by Boyd and Hamilton[13, 19] in England leads to conclusions in closest harmony with those eached at Carnegie. Boyd and Hamilton likewise regard the Bumm and panner theories as untenable in the light of current findings and, furthermore, hey express their conclusions in terms so universally applicable that they merit epeated quotation. "Our view . . . that the arteries and veins open into or ut of the intervillous space more or less regularly over the whole surface of the asal plate . . . has not the functional appeal of the interpretations of either umm or Spanner. This disadvantage is much more than compensated for y the fact that it corresponds with what is actually found in well-fixed sections f placentae *in situ*. The need for a functional explanation of the circulation n the intervillous space is hardly met by postulating an arrangement of the essels which however useful in explaining the flow has actually no basis in natomical fact!"[19]

However, before discarding Bumm's and Spanner's theories, it is both fair nd instructive to consider how these investigators may have reached their onclusions. FIGURE 6*a*, which was presented as evidence of basal entry of rteries, by fortunate chance displays also a maternal septum containing tissue paces (at *X*) such as Bumm mistook for maternal arteries. Remnants of lands, lymphatic channels, or localized foci of liquefaction necrosis may occasion uch deceptive configurations; on the other hand, in some instances the blind xcursion of a vessel into a maternal septum (doubtless drawn upward with urrounding decidua by retraction of an anchoring villus) may cause similar onfusion. Injections, however, clearly demonstrate that there are no arterial ntries to the intervillous space on the septa. It seems likely that in Spanner's ase the heavy injection medium (celluloid) occluded the slender, oblique central enous drainage channels (FIGURES 6*b* and 6*c*) while accumulating in certain ider, more direct marginal channels (FIGURF 7*b*). The false impression thus reated could not be corrected by histological examination, since Spanner diested all fixed tissues to prepare the celluloid cast of the vascular system and the tervillous space (see FIGURE 7*a* and its legend).

FIGURE 6. (*a*) The base of the attached human placenta with orifices of two placental areries (*A* and *B*) and a placental vein (*C*). The absence of short-cutting between artery *A* nd the venous orifice to which it is immediately contiguous is striking, the more so as the resence of villi drawn down into the mouth of the vein indicates active drainage into it. ote that the arteries enter the intervillous space at the base of the septum, not along its des or at the summit. At *X* are seen examples of the tissue spaces which Bumm may have istaken for arteries. Human 8991. Twentieth week of pregnancy. Slide B 30. ×8⅓.) A portion of the trophoblastic plate showing a venous exit from the intervillous space that pplies two maternal veins, one coursing to the right by a narrow, almost horizontal channel, e other to the left. The orifice of exit is located immediately below the point of attachment the umbilical cord. Human 9033. Term. Slide A 200. ×25. (*c*) A similar exit from e same placenta as that shown in 6*b*. The orifice in the trophoblastic plate again lies in e region of attachment of the cord, 5.25 mm. distant from the orifice in *b*. Human 9033. erm. Slide A 95. ×25. FIGURES 6*a*, 6*b*, and 6*c* reproduced from Ramsey.[23]

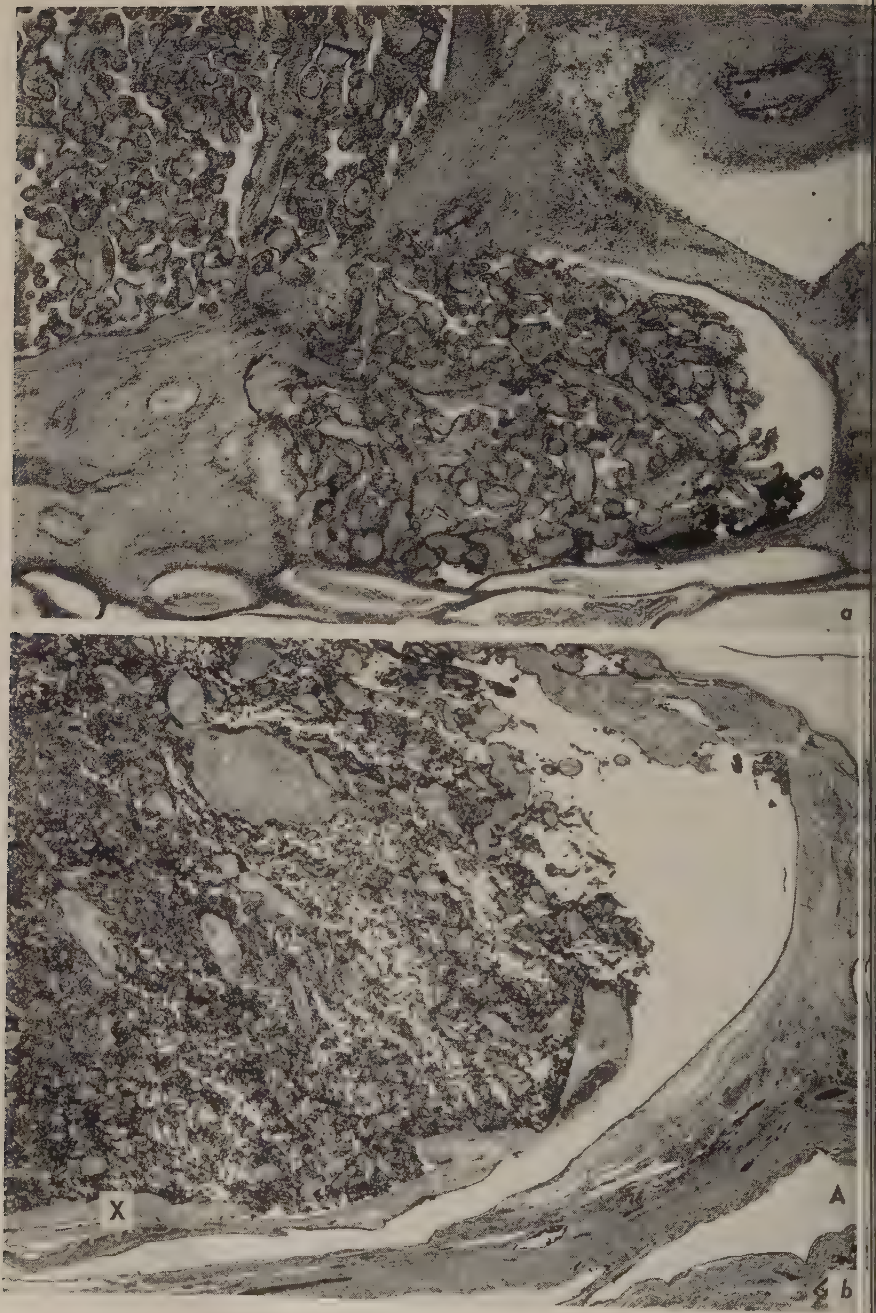


FIGURE 7

It is impossible at the present time to accord to the "marginal sinus" of
panner quite so pre-eminent a position in placental circulation as he did, but
ere is reason to believe that the space does exist (see Hamilton and Boyd[19] to
e contrary) and functions usefully, though most modern workers would wish
see it carefully defined and perhaps called by another name. "Sinus"
nnotes a circumscribed channel with definite walls and continuity, whereas
point of fact the given area is not a separate channel, but merely the triangular
eripheral rim of the intervillous space itself, continuous with the subchorial
ke above and merging gradually with the rest of the intervillous space centrally.
haracterized by paucity of terminal villous twigs, as the subchorial lake is also,
is bounded laterally by the chorion as it dips down to become applied to the
ecidua vera and below by the decidua basalis (FIGURE 7*b*). Its most interesting
aracteristic is its discontinuity, certainly in space, perhaps also in time.
metimes no such structure is demonstrable through half the circumference of
given placenta; then small localized dilatations of the margin of the intervillous
ace may appear. To such intermittent areas the name "marginal lakes" seems
rticularly applicable.[2,3] The fact that they are best seen in distended,
situ placentas (versus delivered specimens) indicates their fluctuation in
rms of blood content and suggests that they may function as safety reservoirs
cope with temporary overfilling of the placenta, resulting from abnormal
cess of blood or impeded drainage.

Fortunately something constructive emerges from these recent studies, on the
sis of which a dynamic and physiological concept of placental circulation can
evolved to replace the older theories. This concept may be represented
agrammatically, as in FIGURE 8*a*, and formulated as follows: arterial blood
ters the placenta from the endometrial arteries under a head of maternal
essure sufficiently higher than that prevailing in the vast, amorphous lake of
e intervillous space, so that the incoming stream is driven high up toward the
orionic plate. Gradually this force is spent and lateral dispersion occurs,
ded by the villi which, acting as baffles, promote mixing and slowing and, by
eir own pulsation, effect a mild stirring. Eventually the blood in the inter-
llous space falls back upon the orifices in the basal plate that connect with
aternal veins and, since there is an additional fall in blood pressure between
e intervillous space and the endometrial veins, drainage is accomplished. The
essure differential is further enhanced by the intermittent myometrial con-
actions (Braxton Hicks) which compress the thin-walled veins, temporarily
eventing escape of blood from the placenta and raising the intervillous pressure.

FIGURE 7. (*a*) The trophoblastic plate showing a venous exit from the intervillous space
a point 41 mm. central to the placental margin. A large orifice of this type would not be
cluded by a heavy injection mass in the intervillous space, but might be confused with an
ery if fixed tissues were digested away. Human 9105. Seventh month of pregnancy.
de B 80. ×12. (*b*) The margin of the attached placenta showing dilatation of the
iphery of the intervillous space to form a marginal lake, drained in the present instance by
vide venous channel. A slender vein with oblique course drains the marginal lake in-
ectly via an orifice in the trophoblastic plate at *X*. At *A* is a part of the wreath of maternal
ns which characteristically lies beneath the base of the placenta. Human 9033. Term.
de D 50. ×10. FIGURE 7*b* reproduced from Ramsey.[23]

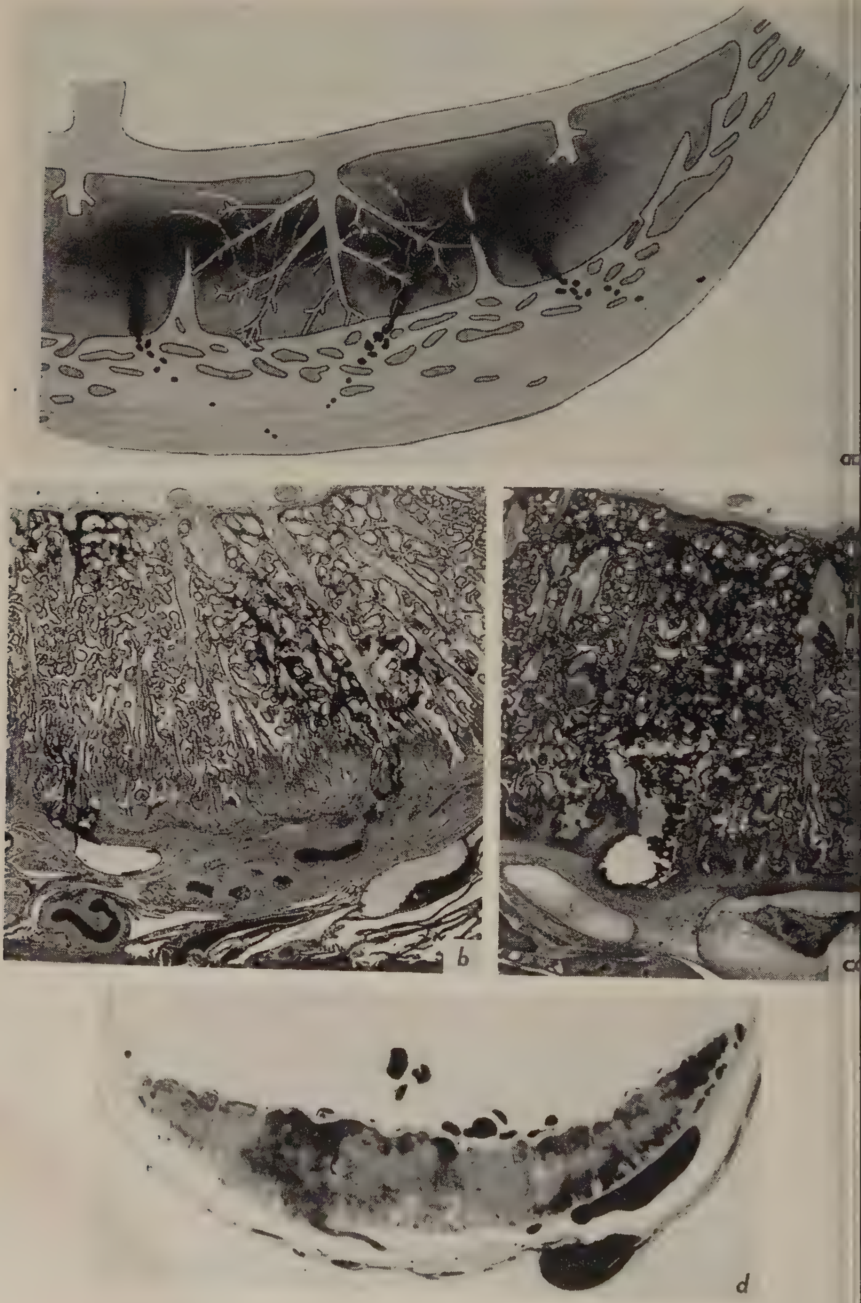


FIGURE 8

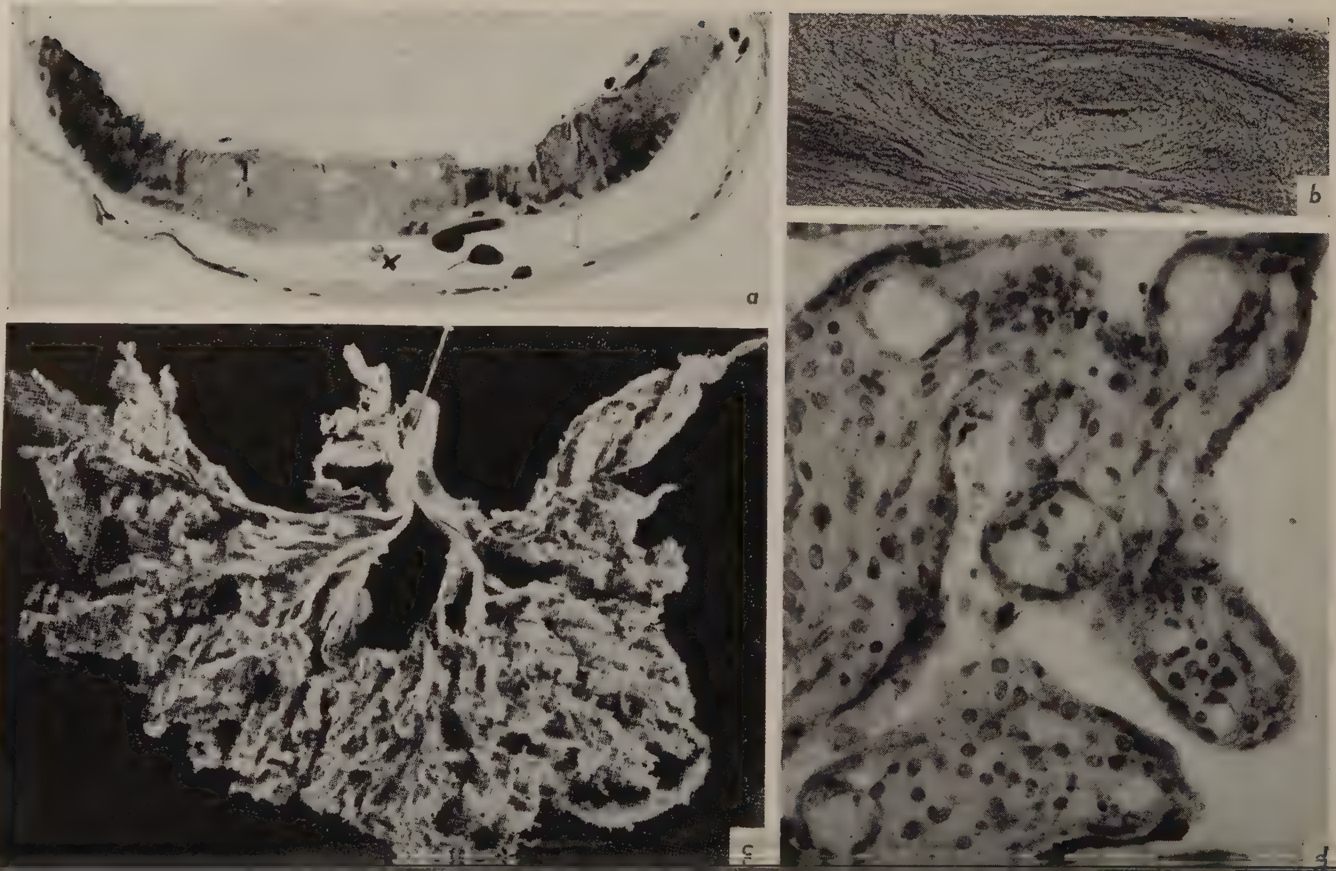
'hen the myometrium relaxes, the elevated intervillous pressure produces pid drainage. It should be noted that this action of the myometrial con-actions is the antithesis of that ascribed to them by Grosser[29] and others who ıvisaged the placenta being "squeezed like a sponge" and its content of blood pressed.

In the above concept, it is seen that pressure differentials rather than morpho-gic arrangements effect the separation of afferent and efferent streams and event short-cutting. An ancillary safeguard against the latter doubtless s in the appreciable distance now known to lie between most of the orifices the basal plate (FIGURE 4).

Representative examples of the anatomical findings that gave rise to the con-pt described are shown in FIGURES 8*b*, 8*c*, 8*d* and FIGURE 6*a*. The spurts of jection mass seen rising from their points of entry in the basal plate bear stimony to the action of the head of maternal blood pressure propelling them IGURES 8*b*, *c*, and *d*). No short-cutting is seen; instead, venous streams ac-ımulate in the subchorial lake or high in the intervillous space and converge dependently upon basal venous orifices of exit, as seen in FIGURES 6*a* and 8*b*.

Significant evidence of the uniformity of arterial behavior throughout the enstrual cycle and pregnancy is shown by the vessel in FIGURES 9*a* and *b*. he wide band of uninjected intervillous space conspicuous in FIGURE 9*a* is pplied by an artery whose entry is seen in a section 500 μ deeper than the one ustrated. At X in the section shown, this artery is seen in the endometrial ongiosa, its lumen patent but as devoid of injection material as the communi-ting area of intervillous space. The state of the vessel at the level shown in GURE 9*b*, 5 mm. removed, provides the explanation of these phenomena. the level of FIGURE 9*b*, the artery has penetrated the myoendometrial junc-ɔn, its wall is tightly constricted and the lumen occluded, barring passage to jection material. Since this is an autoinjection specimen (see page 733), it in fact the normal, physiologically circulating blood that is thus barred. ontractility such as this artery shows and the independence of its activity e likewise prime characteristics of Type I arteries in the menstrual cycle. In ldition, this example of nonfilling of a single arterial stem permits evaluation the size of the area of intervillous space supplied by a single placental artery.

FIGURE 8. (*a*) Diagrammatic representation of the course of circulation through the ervillous space of the placenta. Arterial blood entering through orifices indiscriminately ıttered over the basal plate is driven by the head of maternal pressure high up toward the orionic plate before lateral dispersion occurs. After bathing the chorionic villi the blood nverges upon venous orifices in the basal plate and enters the maternal placental veins. India ink injection of a monkey placenta via abdominal aorta. Maternal arterial blood ırts into the intervillous space on the right. A maternal placental vein drains ink from subchorial lake through an orifice in the basal plate at the center of the photograph. onkey C 658. Thirty-eighth day of pregnancy. Primary placenta. Section 34. Hema-xylin and eosin. ×10. (*c*) India ink injection of a monkey placenta via abdominal aorta. aternal arterial blood entering the intervillous space through an orifice in the basal plate left spurts upward to the chorionic plate with minimal lateral diffusion. Monkey C 629. 'ty-third day of pregnancy. Secondary placenta. Section 52. Hematoxylin and eosin. 10. (*d*) Autoinjection of a monkey placenta. Bouin's bath of *in situ* uterus. Maternal ɔod, colored by contact with Bouin's solution (see p. 733), spurts high into the intervillous ıce from an arteriole on the left. Monkey C 750. One hundred and twenty-third day of gnancy. Primary placenta. Section 87. Unstained. ×1. FIGURE 8 reproduced from msey.[25]


a
b

The physiological theory of placental circulation is confirmed and im-lemented by the data on absolute pressure values obtained by investigators vho have recently concerned themselves with such determinations. Woodbury nd his co-workers[30] in 1938 did the pioneer work along this line. Alvarez and Caldeyro-Barcia[31, 32, 33] have been active throughout the present decade, and he past two years have seen a number of new entries into the field.[34, 35, 36] Reports of findings by several investigators of intrauterine pressure appear lsewhere in these pages. To date the Carnegie monkey experiments appear o be the only ones conducted in a primate other than man.[25] Results so far ave been gratifyingly uniform in confirming that the head of maternal arterial ressure is about 60 to 70 mm. Hg greater than that prevailing in the intervillous pace and that the latter in turn exceeds the 8 mm. of venous pelvic pressure by everal millimeters even during myometrial diastole. Caldeyro-Barcia[32] ecords normal average myometrial contractions up to 80 mm. Hg, and all nvestigators agree that amniotic and intervillous space pressures rise concomi-antly. There is some diversity among the absolute values reported for various f these pressures, but accumulation of further data may be expected to adjust he discrepancies since ranges and relationships harmonize well and all support he validity of the dynamic, physiological concept of placental circulation.

It may seem to be an unjustified stretching of the categories to incorporate onsideration of fetal circulation in a discussion of uterine vasculature in preg-ancy, but the intimate interlocking of the two, anatomically and physiologi-ally, renders the decision to include at least a brief précis a logical one. Indeed, he story of placental circulation is only half told otherwise.

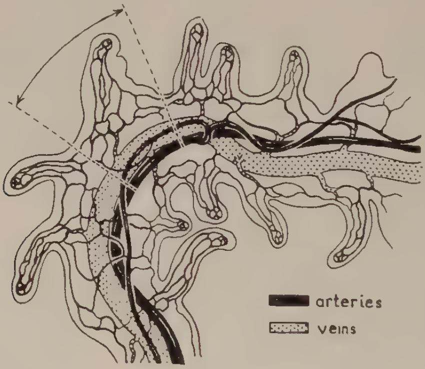
Essentially the purpose and *raison d'être* of the intervillous space is to form pool of maternal blood in which fetal villi, containing fetal blood vessels, may e immersed under circumstances maximally conducive to exchange of gases nd electrolytes between the two blood systems.

In the primate, this is achieved by the developments on the maternal side lready outlined. On the fetal side, a variety of study techniques have shown Bøe,[15,16] Crawford,[37-40] Danesino,[41] Javert,[42] Lemtis,[43] Paine,[44] Romney,[45] Vilkin[46]) that villi arising from the chorionic plate, where their roots may be aid to grow, depend into the intervillous space like the trunk and spreading mbs, branches, and twigs of a deliquescent tree (FIGURE 9*c*). Many of the

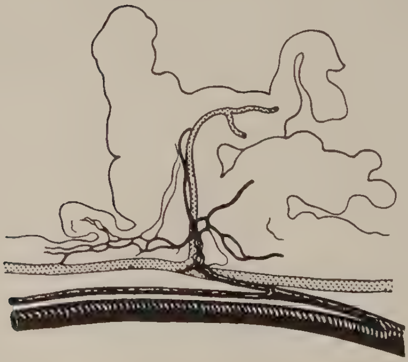
FIGURE 9. (*a*) Autoinjection of a monkey placenta. Bouin's bath of *in situ* uterus. Note rge central anemic area of intervillous space. A patent cross section of the arteriole sup-lying this area is seen at *X*. Monkey C 750. One hundred and twenty-third day of preg-ancy. Secondary placenta. Section 123. Unstained. ×1½. Reproduced from Ram y.[25] (*b*) The same specimen as that seen in 9*a*, 5 mm. removed. The arteriole supplying ie anemic area is here seen as it crosses the endometrial-myometrial junction. Its complete cclusion by vasospasm explains the bloodlessness of the area of intervillous space which it ipplies. Section 113. Unstained. ×45. Reproduced from Ramsey.[25] (*c*) "A cotyledon is been digested and the subcotyledons sufficiently separated to show their origin from the ain trunk."[38] The paucity of terminal villi arising from the main stems is apparent, as is the oundance of such units in the two thirds of the placenta nearest the decidual plate. Re-roduced by permission from Crawford.[38] (*d*) Fetal capillaries in a human placenta are en close to the periphery of terminal villi. The fetal blood and maternal blood at such oints are separated only by capillary endothelium and syncytial trophoblast. Flecks of in-cted India ink are seen in the intervillous space on the left. Human 8593J. Twenty-cond week of pregnancy. Hematoxylin and eosin. ×400. Reproduced from Ramsey.[25]

terminal twigs merge with the decidua as anchoring villi (see in particular th extensive recent studies of Crawford[37-40]). For the "weeping willow" configuration of Spanner,[12] there is little generally accepted evidence at th present time.[39, 45]

In the treelike ramifications of the villi injections have demonstrated tha long arteries and veins of relatively wide caliber course through the trunk and stem down toward the terminal branches, the arteries frequently spiraled.[4 Danesino[41] has described arteriovenous anastomoses between the larger vascula stems close to the chorionic plate, as well as certain "beads" in the intima o stem veins caused by subendothelial nodules of muscular tissue, but these findings await confirmation by others.



Superficial capillary network



Paravascular network

FIGURE 10. Schematic representations of the course of fetal blood vessels in the chorionic villi of the human placenta, after Bøe (1953). In the upper drawing the vasculature is shown as it would appear if the paravascular network had been dissected away. The points at which this network would attach to the stem vessels are indicated by the broken lines. Reproduced from Ramsey.[22]

Within the twigs that represent the terminal absorptive units of the vascular ree is lodged the all-important capillary bed. Romney's[45] vinylite casts demonstrate that the precapillary arterial bed is extremely intricate and highly ramified, and that the arteriolar tips are seldom separated by more than 1 mm. rom the beginning of the connected venules. Finn Bøe[15] found two types of capillary complexes occupying this 1 mm. interval (FIGURE 10); the first, a superficial network within the terminal villi; the second, a paravascular network providing extravillous shunts from artery to vein that act as safety valves to prevent overloading of the villous circulation (see Ramsey's comment on Bøe, (954[16]). Crawford noted only the superficial network, which he described in detail.[39] Crawford,[39] Javert,[42] and Paine[44] noted that in the mature placenta etal capillaries frequently lie immediately subjacent to the syncytial covering of the villi. The relationship is readily observed in most normal, mature placenas (FIGURE 9*d*), and probably these "placental sinusoids" represent, to quote Paine, "a response to the increasing gaseous exchange requirements of the oetus."[44] The formation has important physiological and pathological implications for fluid and electrolyte exchange and for protein sensitization, since t represents a thinning of the placental barrier to two delicate unicellular membranes.

The circulatory mechanism on the fetal side of the placenta has an important eature in common with that of the maternal: namely, that the *vis a tergo* of etal blood pressure dominates it just as the head of maternal pressure dominates the maternal side. Barcroft,[47] Barron,[48] Dawes,[49] and Reynolds[50] have demonstrated the special features of the fetus' own circulation by virtue of which arterial blood reaches the placenta at a relatively high pressure. Because of the unusual width of the arterial channels in the villous stem vessels, little pressure is lost by friction before the capillary networks are reached. At the ame time pressure is kept high on the venous side, again by circumstances operating within the fetal body. As a result, Reynolds finds that the fall in pressure between umbilical artery and umbilical vein is only some 35 to 40 mm. Hg. Capillary pressure, therefore, is high, greatly exceeding the resting pressure in the intervillous space, with significant consequences for fluid exchange.

In conclusion, the thesis of the opening paragraphs may be restated and reffirmed. The potentialities for all the changes exhibited by uterine vasculaure during pregnancy exist in the vessels at all times. Three basic ones characerizing the Type I (spiral) arteries may be regarded as the pivotal ones upon which all endometrial alterations depend, directly or indirectly: (1) potentiality or growth under hormone stimulation; (2) potentiality for vasoconstriction when hormone levels drop; and (3) potentiality for independent activity with, s a corollary, variation in susceptibility to hormone stimulation—arteries upplying the implantation sites being the more sensitive.

Operation of these basic potentialities occasions the phenomena of all phases f the "reproductive cycle," the latter term being considered a more fitting xpression of the smooth, unbroken, purposeful sequence of changes inaugurated n day 1 and, if an ovum implants, continuing through to term.

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THE DYNAMICS OF THE AMNIOTIC FLUID

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Over the past five years the investigations of my associates and myself have been concerned with the study of over-all exchange mechanisms between the products of gestation and the maternal organism. The stimulus to this project was the pioneer work of Flexner and his associates on the transfer of water and sodium in the pregnant organism. By means of deuterium oxide and sodium-24, these investigators measured the transfer rates of water and sodium from the maternal organism to the fetus and also the transfer rates from the maternal organism to the amniotic fluid. The "backflow" or other exchange mechanisms were not considered in their calculations. It was assumed, however, that an equal amount of the transferred species would be returned that was not and could not be substantiated under the conditions then prevailing. Despite this shortcoming, the formulas used by Flexner, admittedly an approximation, were thought to be applicable. The analytic values carried a fairly large error, in some cases nearly 100 per cent, with no possibility of determining their magnitude since, for each individual patient, only one value for the tracer concentration could be obtained. Such data may be informative if based on a sufficiently large number of subjects of comparable age, parity, and length of gestation; Flexner's data, however, are based on 5 patients whose gestational ages ranged from 14 weeks to term. These authors' final conclusion that 34 per cent of the water of the amniotic fluid is exchanged per hour implies that the rate of equilibration for the amniotic fluid is considerably slower than that for the maternal organism. It was our thought that this difference should enable us to calculate transfer rates with a much higher degree of accuracy and to use the correct mathematical treatment of the data.

The problem of water exchange of the amniotic fluid assumes considerable clinical significance under the pathological conditions of hydramnios and the rare oligohydramnios. The volume of the amniotic fluid varies throughout pregnancy for individual patients, and term pregnancies may be associated with amniotic fluid volumes ranging from a few hundred to several thousand milliliters. It would be desirable to know whether the exchange is effected in any manner and what its relation to the variability of the amniotic fluid might be. A comparison of normal and abnormal conditions cannot be based on the approximations used thus far, but requires much more precise and accurate measurement of transfer rates. Improvement in tracer analyses and techniques should lead to a higher degree of precision, and the more recent theoretic developments in the field of multicompartment systems provide the basis for the calculation of the backflow from a minimum of data.

For reasons of expediency, our original studies were concerned only with the exchange between mother and amniotic fluid. This admittedly simplified system lends itself well to a mathematical analysis. In order to investigate this simplest transfer mechanism, isotopes were injected into the mother or the amniotic fluid, and their disappearance or appearance was measured as a function of time. The mathematical background for this has been discussed in some detail in preceding reports[1-3] and need not be repeated here. If the fetus and placenta influence the exchanges between the mother and amniotic fluid, the simple two-compartment exchange mechanisms no longer suffice. For a more detailed investigation of this problem, a fairly complicated three-compartment system must be assumed. To prove or disprove the correctness of such assumptions, time-concentration curves for all three compartments must be determined experimentally. Samples of amniotic fluid and maternal and fetal body fluids should be analyzed; only from such data could true transfer rates for all compartments be calculated. In view of the inaccessibility of the human fetus, indirect evidence must supply the answer.

The Two-Compartment Case

Transabdominal catheterization makes the amniotic fluid accessible for sampling.[4] Tracers can be injected into this compartment, and time-activity

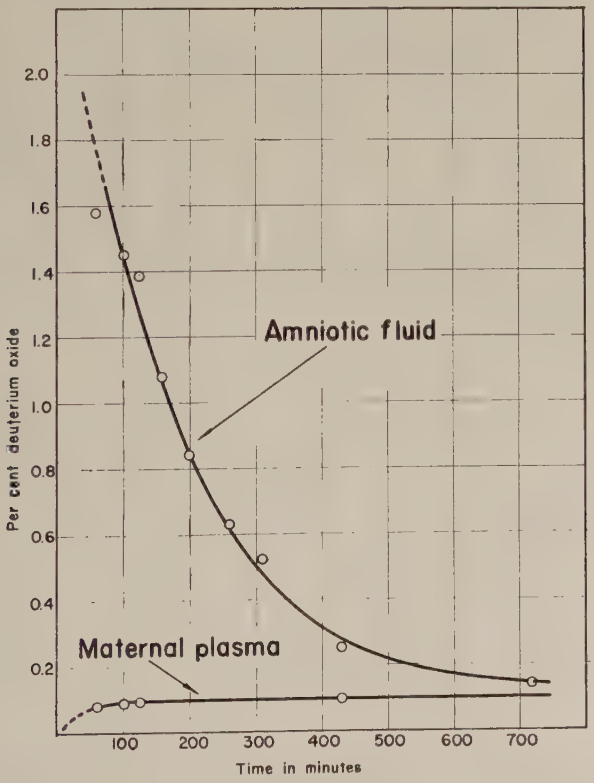

FIGURE 1. Changes in deuterium oxide concentration of amniotic fluid and maternal compartments as a function of time. Cartesian coordinates.

curves can thus be constructed. For simple exchange mechanisms, the theory predicts that exponential curves will be obtained for maternal and amniotic fluid pools. From these curves an accurate estimate of the transfer can be made. A typical experiment is illustrated in FIGURE 1, where deuterium oxide had been injected into the amniotic sac and its disappearance measured over a period of several hours. The tracer concentration in amniotic fluid shows the anticipated decline and, conversely, a rise of tracer concentration in maternal plasma was recorded. Curves of similar shape were obtained on a variety of patients who were comparable in gestational age, but differed in the volume of the amniotic fluid, maternal weight, and total body water.

Over their major portion, these curves are characteristically exponential and, if the data are plotted on semilog paper, a straight line should be obtained. The slope of this line is proportional to the percentage of the species exchanged, and its dimensions are expressed as a number per unit of time. FIGURE 2 shows this relation for three patients whose amniotic fluid volume differed widely. The two extremes of oligohydramnios and polyhydramnios are compared with

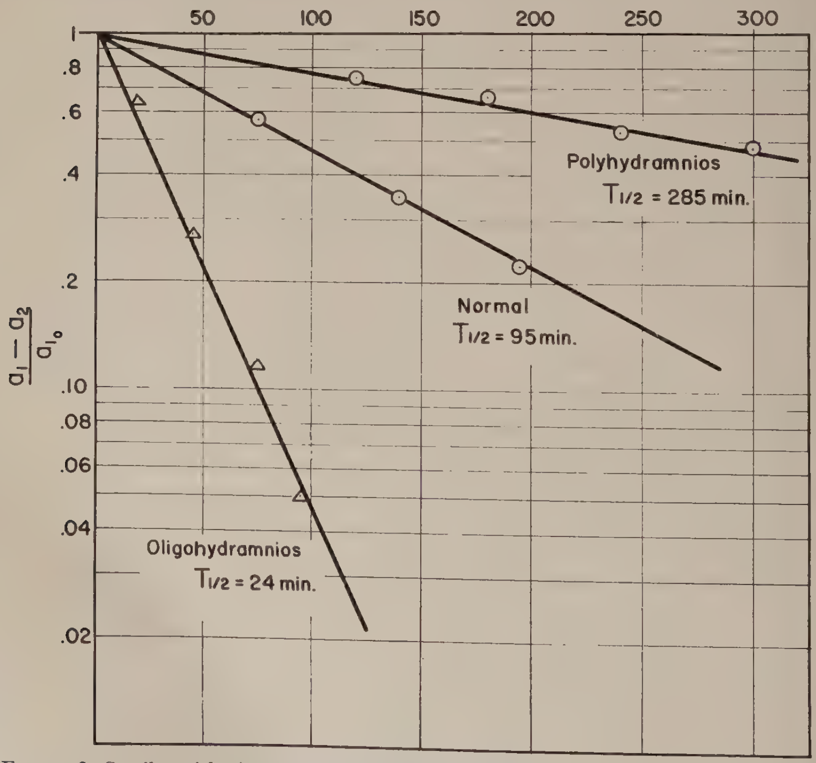

FIGURE 2. Semilogarithmic plot of tracer concentration against time for term patients with different amniotic fluid volumes. The slopes are disappearance constants, and T ½ is the biological half lifetime.

normal patient at term. The slopes for these patients show a striking fference, and one might be tempted to conclude that this difference is a flection of a disturbance in water exchange. The numerical value for the ope is inversely proportional to the volume: the larger the volume (polyhy- ramnios), the smaller the slope (fraction exchanged per unit of time). An ccurate knowledge of the volume of the amniotic fluid and the proportionality ctor makes it possible to calculate the absolute quantity transferred per nit of time. If this is done, one finds that, within the limits of error, this uantity is identical for all three conditions. This was determined on 36 ccessive patients, and a mean value of 26 moles per hour was found.

In TABLE 1 the amniotic fluid volume is correlated with the transfer of water. atients with amniotic fluid volumes of less than 2000 ml. exchanged 26.1 oles, while those regarded clinically as hydramnios exchanged 27.3 per hour. tatistically the difference is not significant. The total amount of water ex- anged is therefore independent of the volume of the amniotic fluid.

Using essentially the same approach, we can study any other traceable ement. The quantity of sodium and potassium can be measured separately d simultaneously by using isotopes that do not interfere in analytic pro- dures. In the calculation of the transfer rates of these elements, the same rmulas are used, but the volumes of the respective compartments refer to e total amount of the traced isotope and not the volume of the amniotic uid. The average values for transfer rates of sodium and potassium are ven in TABLE 1. They are nearly identical for normal and hydramniotic atients; about 12 mEq. of sodium and 0.5 mEq. of potassium are exchanged er hour.

That the exchange mechanisms for different elements are entirely inde- endent of each other is shown by the fact that the ratio of the transfer rates r sodium and water and potassium and water is different from the ratio of eir respective concentrations. The transfer of 12 mEq. of sodium would re- uire about 55 moles or 55,000 mEq. of water, while actually only one half at much water is transferred per hour (TABLE 2). Since this is beyond the perimental error, one can conclude that the constituents of the amniotic id exchange at their own characteristic rates. The amniotic fluid is not an trafiltrate of serum, but a pool of a variety of components, each of which is

TABLE 1

RELATION OF AMNIOTIC FLUID VOLUME TO TRANSFER RATES FOR WATER, SODIUM AND POTASSIUM

Amniotic fluid volume	Transfer rates (mEq./hour)		
	D_2O	Na	K
ss than 2,000 ml. (range 385–1420)	26,100	12.0	0.5
ore than 2,000 ml. (range 2,000–6,000)	27,300	12.4	0.6

The patients were divided arbitrarily into two groups; 2000 ml. was considered the viding line between normal and abnormal states.

TABLE 2

COMPARISON OF THE RATIO OF CONCENTRATIONS TO RATIO OF TRANSFER RATES FROM
AMNIOTIC FLUID TO MOTHER FOR SODIUM AND WATER

$$\text{Ratio of concentration} = \frac{55{,}555 \text{ mEq. } H_2O}{125 \text{ mEq. Na}} = 0.44 \times 10^3$$

$$\text{Ratio of transfer rates} = \frac{26.600 \text{ mEq. } H_2O}{12.5 \text{ mEq. Na}} = 2.13 \times 10^3$$

The figures represent average values for all patients.

in a well-ordered state of equilibrium with its surroundings; that is, the materna organism and probably the fetus.

The Three-Compartment Case

Without specifying the pathway or manner in which it is accomplished, th transfer rates from mother to amniotic fluid and in the reverse directio have been determined with reasonable accuracy. This defines only the transfer between the two compartments and does not give any information o the participation of the fetus. Water can be transferred from the materna organism to the amniotic fluid directly or it may enter the fetoplacenta circulation and from there be secreted or diffused into the amniotic flui Similarly, the water of the amniotic fluid may go directly to the mother o on its way, pass through the fetus. All these possibilities must be considere in the application of theories designed to determine the magnitude of th exchanges.

If the fetus participates in these exchanges, the system is composed of minimum of three compartments, all of which, at least potentially, exchang with each other. Compared to the transfer of water from the mother t the amniotic fluid, the transfer of water from the maternal organism to th fetus is very rapid, and it is this difference that made it possible to determin the latter transfer with the accuracy described above. Since neither fetus no amniotic fluid shows an appreciable change in volume during the brief experimental periods, it can be assumed that an equal amount of water is transferre in the opposite direction; that is, from fetus to mother. The transfer from the amniotic fluid to the mother has been demonstrated experimentally, but the pathway, even in its broadest terms, has not been defined. If the tracer is injected into the amniotic fluid and appears in the maternal body fluids, it may have gone there directly or passed through the fetus. The results are compatible with either possibility.

Although a knowledge of the rate of replacement of the amniotic fluid water is important, it does not, as has been shown, bear any obvious relationship to the quantitative variability of the amniotic fluid. Investigations on the role of the fetus would seem to be more promising, since clinicostatistical and epidemiological studies have shown a certain relationship to pathological states of the fetus. For this reason, the role of the third compartment, the fetus, is of considerable clinical and scientific significance.

The measured transfer rates can be expressed schematically as follows:

Scheme I — or — Scheme II

$$\begin{tikzcd} & \text{FETUS} & & & \text{FETUS} \arrow[dr] & \\ \text{MOTHER} \arrow[ur] \arrow[r] & \text{AMNIOTIC FLUID} & \text{or} & \text{MOTHER} \arrow[ur] & & \text{AMNIOTIC FLUID} \end{tikzcd}$$

The experimental data cannot be used to decide which of the two schemes s applicable. Injection of the tracer into the maternal compartment would not, on theoretical grounds, yield any information on that point. Injection of the tracer into the amniotic fluid compartment could, under certain circumstances, permit a very rough estimation of the magnitude of this exchange; n particular, whether the second scheme is possible. When deuterium oxide s injected into the amniotic cavity and the experiment is terminated within a period of less than one hour, a surprisingly high concentration of the tracer s found in the fetus. In fact, the concentration of the tracer in that compartment exceeds the calculated equilibrium value (the tracer concentration at nfinity) for that tracer. This finding indicates that a time-activity curve for he fetal compartment must go through a maximum, since its initial value is ero and its final value is equal to the calculated equilibrium value. Such a hape is possible only if Scheme II is applicable. It can be shown further that t least 25 per cent or more of this transfer from amniotic fluid to mother must ake place through the intermedium of the fetus. The theoretical proof for his has been reported previously and need not be repeated here.[3]

Even though these estimates could be narrowed still further by additional xperiments, they would only give specific information on one additional ransfer rate in this complicated system. At this point, therefore, the following ypothetical transfer rates are known within a wide margin of error:

$$\begin{tikzcd} & \text{FETUS} \arrow[dl, dashed, shift right] \arrow[dr, dashed, shift left] & \\ \text{MOTHER} \arrow[ur, shift right] \arrow[rr, shift right] & & \text{AMNIOTIC FLUID} \arrow[ul, shift left] \arrow[ll, dashed, shift right] \end{tikzcd}$$

The solid arrows represent measured rates; the dotted arrows designate transers that may exist, but have not been verified experimentally. Each of these ransfer rates may vary widely from patient to patient, despite the fact that he patients themselves may be comparable in every respect. This represents highly individual set of circumstances, and an attempt to evaluate the remainder of the transfer rates on a statistical basis would be futile. One whole ystem of transfer rates must be determined on one patient; only such a completed picture can be compared to that of another patient. The simplest ossible way in which such a system can be represented is a three-compartment xchange between fetus, mother, and amniotic fluid. Since there are three ompartments, each exchanging with the other two, there are six possible ransfer rates. To determine the value for each was the aim of the present tudies.

The application of differential equations to this particular biological system is limited by a number of circumstances. It should be possible to predi the type of experiment likely to yield the desired information. This is importance because we are dealing with three compartments of striking different size (the maternal fluid compartment is roughly 25 l. compared to 1 for amniotic fluid and fetal compartments). This same circumstance al made it possible to ignore the fetus in the original exchange studies and calculate with reasonable accuracy the quantity exchanged between amnio fluid and the other compartments. This, in turn, is due to the fact that t exchange rate between mother and fetus is many times faster than the exchan rate between mother and amniotic fluid, and it is the slowest exchange ra which represents the rate-determining step.

In order to accumulate as much information as possible and to be able define the experiments as precisely as we could, hydrodynamic models whi yielded hypothetical time-activity curves for the three compartments we constructed.[2] Such information, of course, could be obtained on purely theoret cal grounds but, since an electronic computer was not available to us, th studies on models were the only alternative. The problems to be decide in advance related to the site of tracer injection and the optimal time for term nation of the experiment. The minimum requirement for reconstruction the fetal time-activity curve is two points in addition to zero and the calculate equilibrium value. If only one point intermediate between zero and equ librium is available, an infinite number of curves can be drawn, and such datum would yield no useful information. Two points for the fetal curve coul be obtained if 2 tracers for the same element (here deuterium and tritium were injected into the same compartment but at different times. The resul obtained on the hydrodynamic model showed that injection of the trace into the maternal compartment would not permit us to calculate transfer rat with any acceptable degree of accuracy. If the tracer is injected into th amniotic fluid compartment, transfer rates can be calculated satisfactoril provided the proper time intervals are chosen. Injection of the tracers int the fetal compartment is, of course, excluded because of the inaccessibilit of the fetus.

Such experiments would be based on a number of assumptions that requir some clarification. In addition to the main assumptions governing all trace experiments,[5] the system (here the pregnant maternal organism) must b assumed to be in a steady state. The term steady state is applied to compar ments where the rates of removal of the substances under investigation ar equaled by their rates of replacement, so that the concentrations and amount of the substances being studied are constant during the period of observatio Since no evidence to the contrary exists, the assumption of a steady state i perfectly valid under the circumstances. Also, it is assumed that the tracer used will perform in identical manner at least over the brief period of observation. The tracers to be used were deuterium and tritium and, because of th wide difference in mass of hydrogen, deuterium, and tritium, an isotope effec conceivably could interfere. To demonstrate the validity of this assumption, a series of clinical experiments was performed in which first deuterium nd then tritium was injected into the amniotic sac of pregnant patients at erm. The disappearance of these compounds from the amniotic fluid and heir appearance in the maternal circulation were measured, and it could be hown that the two tracers behaved in an identical manner. One such experinent is illustrated in FIGURE 3, which shows quite clearly how the tracer curves 'chase" each other, the second reproducing a pattern identical with the first, emporally displaced. An isotope effect is therefore negligible and need not e taken into account.

For all subsequent experiments on clinical material, a standard procedure vas followed. The first tracer, deuterium oxide, was injected 2 to 6 hours efore the planned termination (cesarean section) of the experiment. The

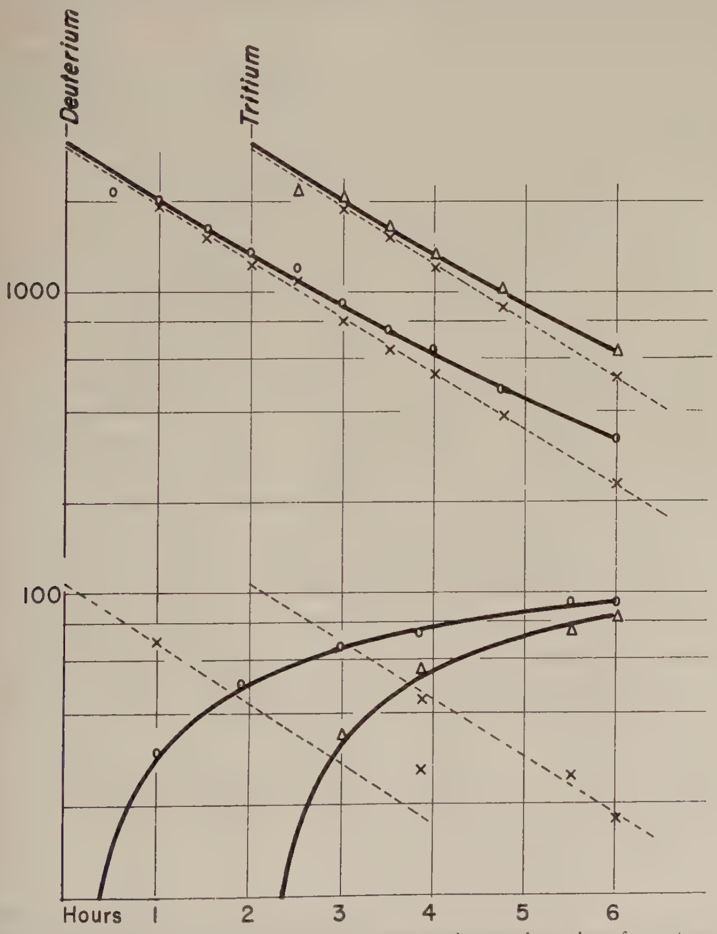

FIGURE 3. Semilogarithmic plot of tracer concentration against time for a term patient vho received first deuterium and then tritium in the amniotic sac. The upper curves are oncentrations of tracer in amniotic fluid; the lower curves are changes of tracer concentration n maternal plasma.

second tracer was injected approximately 15 min. to 1 hour before terminatio The time intervals were so chosen that the concentration of the second trace in fetal blood would reach its peak at about the first half lifetime of the amnioti fluid. At cesarean section, the second tracer would thus fall on the ascendin part of the curve, the first tracer on the descending part. From these tw points and a knowledge of the two exponential constants, the fetal curve coul be reconstructed. The maternal curve was determined purely incidentall but, because of its unreliability, was ignored in the calculations. It usuall fell within the expected range and does not represent a prerequisite for th calculation of transfer rates. This procedure was performed on three norma patients at 30, 38, and 40 weeks. The experimental data and their graphi analysis for one of these is given in FIGURE 4. The calculated transfer rate are reproduced in TABLE 3.

Under conditions of hydramnios, in contrast to the normal, the behavior c the fetal curve seemed unpredictable at first. Model experiments and theoreti cal considerations led to the conclusions that, if the exchange rates were com parable to the normal, the curves for the fetal compartment would be flatte

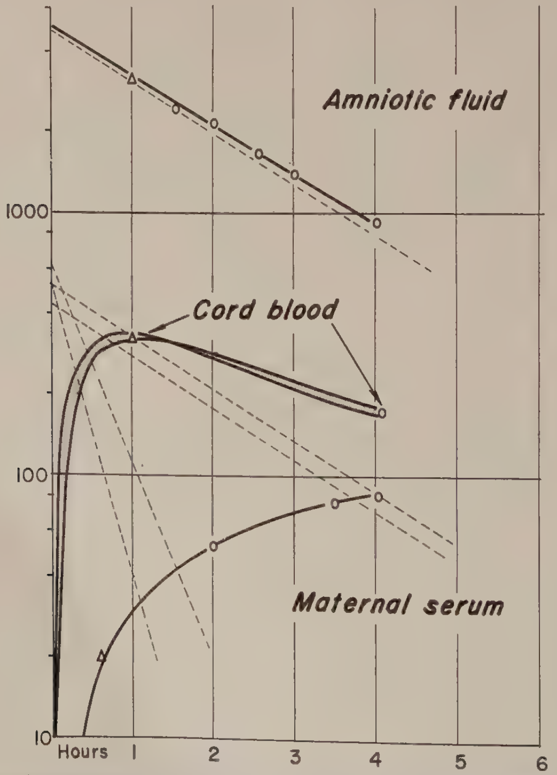

FIGURE 4. Semilogarithmic plot of tracer concentration for the 3 compartments as a function of time in a patient at 30 weeks' gestation. Triangles are tritium analyses; the circles deuterium. The shaded area represents the limits of error for the fetal compartment.

he relative position of the points for tracers 1 and 2 cannot easily be estiated in advance.

Such a study is of value only if cord blood can be obtained at a specific time nd if the condition of a steady state exists throughout the period of the ex-

TABLE 3

TRANSFER RATES FOR WATER DURING LATE PREGNANCIES*

	Thirty weeks	Term	Hydramnios
etus to mother	1475 (1311–1640)	3682 (3648–3716)	423 (420–426)
lother to fetus	1619 (1454–1785)	3657 (3542–3772)	881 (878–884)
.F. to fetus	150 (0–300)	149 (0–298)	563 (561–565)
etus to A.F.	164 (157–171)	165 (150–174)	99 (97–101)
.F. to mother	160 (153–167)	247 (235–259)	527 (524–530)
lother to A.F.	167 (10–324)	265 (97–433)	63 (60–66)

* Mean and range expressed as ml./hr.

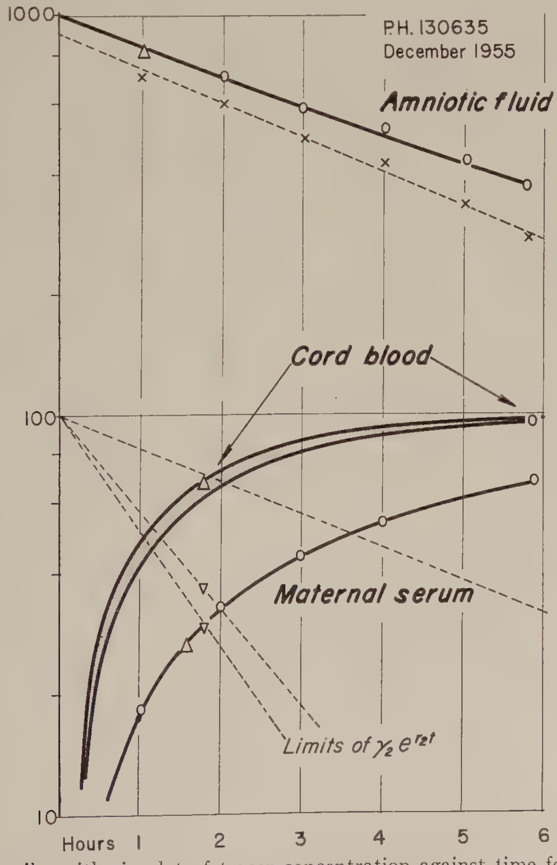

FIGURE 5. Semilogarithmic plot of tracer concentration against time for a hydramniotic atient at term. The shaded area represents the limits of error for the fetal time-activity urve.

periment. Premature rupture of the membranes with an unpredictable delivery time would render the data worthless. This makes an elective cesarean section mandatory. For obstetrical reasons, a section is rarely indicated, and suitable patients are therefore difficult to obtain. Nevertheless, two such cases were studied. The same procedure as that outlined above was followed.

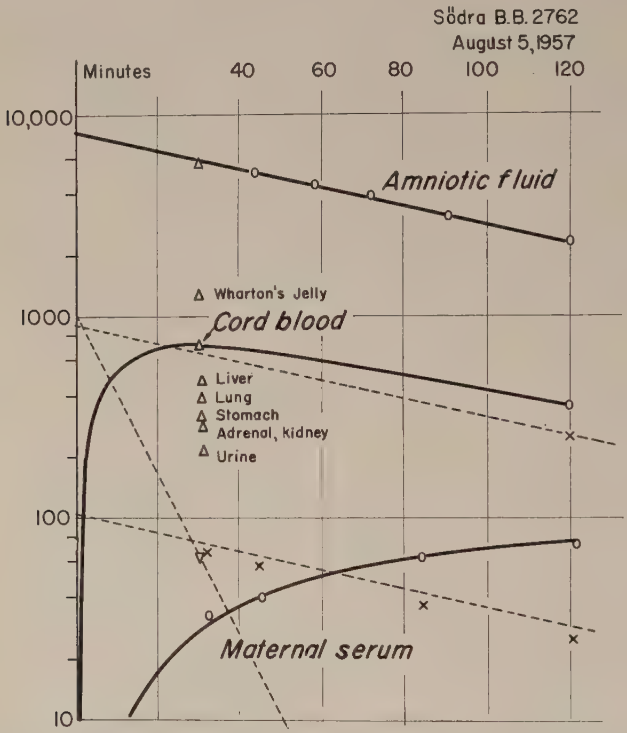

FIGURE 6. Semilogarithmic plot of tracer concentration against time for a patient at 12 weeks' gestation. Hysterotomy was performed 30 min. after the injection of tritium. Relative tritium concentration for various fetal tissues is plotted for comparison.

TABLE 4
TRANSFER RATES FOR WATER DURING EARLY PREGNANCIES*

	No. 2773 12 weeks	No. 2726 15 weeks	No. 2798 15 weeks	No. 2762 20 weeks
Fetus to mother	111 (109–114)	259 (121–398)	222 (80–364)	444 (221–667)
Mother to fetus	100 (98–103)	302 (250–354)	256 (195–317)	459 (360–558)
A.F. to fetus	11 (10–12)	37 (30–44)	38 (29–47)	78 (64–92)
Fetus to A.F.	2.5 (0–5)	70 (0–140)	77 (0–154)	101 (0–202)
A.F. to mother	64 (63–65)	107 (85–129)	97 (73–121)	103 (68–139)
Mother to A.F.	72 (70–75)	79 (0–158)	66 (0–132)	89 (0–178)

* Mean and range expressed as ml./hr.

xcept that the elected time intervals were longer. The results reproduced raphically (FIGURE 5) for one typical experiment indicated that the fetal racer concentration still follows a double exponential curve, but never exceeds he equilibrium value, since both points are below it. Even without a detailed nalysis of the data, this observation suffices to indicate that the fetus, under onditions of hydramnios, does not take as active a part in the water transfer s it does under normal conditions. The calculation of the six transfer rates TABLE 3), which was surprisingly precise, showed a definite impairment of he exchange between mother and fetus. This observation represents the nly striking difference between normal and hydramniotic patients and concides with the frequently expressed belief that the fetoplacental circulation ; impaired in this abnormal state.

Recently the opportunity presented itself to perform similar experiments uring early pregnancies. Here the experimental procedures were changed lightly to take into account the smaller volume of 2 of the compartments. nown amounts of tracers were injected into the amniotic cavity via a catheter ntroduced through the vesicocervical space. A second tracer was injected 5 to 20 min. before termination of the experiment by vaginal hysterotomy. ccurate transfer rates could be calculated for four patients at 12, 15, 15, and

TABLE 5

	No. 2778 12 weeks	No. 2798 15 weeks	No. 2726 15 weeks	No. 2762 20 weeks
ord blood	1380	917	943	700
Vharton's jelly	4546	1762	1616	1276
ung	1018	600	688	463
iver	—	384	505	396
drenal	—	—	492	274
idney	1109	—	618	258
lacenta	386	431	506	315
.S.F.	865	484	—	—
tomach	—	448	550	315
rine	—	322	—	218

TABLE 6

	No. 2773 12 weeks	No. 2726 15 weeks	No. 2798 15 weeks	No. 2762 20 weeks
Vharton's jelly	327	192	171	182
ord blood	100	100	100	100
ung	73	65	73	66
iver	—	42	53	56
drenal	—	—	63	40
idney	79	—	65	37
.S.F.	62	53	—	—
tomach	—	49	58	45
rine	—	35	—	31
lacenta	28	47	58	45

20 weeks of gestation. The final data for these experiments are given in TABLE 4, and the experimental curves for one of them are reproduced in FIGURE 6.

These last four experiments completed this phase of water-transfer studies Application of similar methods for the estimation of sodium and potassium transfers was considered inappropriate for two reasons. First, even unde the most favorable conditions, where all compartments are continuously accessible, as in the pregnant rhesus monkey, and using relatively large amounts of sodium-22, the data obtained were not sufficiently precise to permit calculation of transfer rates. This is probably due to the fact that, in comparison to water, sodium is equilibrated too slowly and that several sodium pools exist. Second, although two radio tracers for sodium are available, the dosage required would come too close to the maximum permissible limit Below this limit the radioactivity measurements of less than "equilibrium' value would be worthless. Such experiments were therefore temporarily abandoned.

In five instances the fetus was available for study; four during early pregnancies, where the operative procedure was done with the intent of terminating the pregnancy, and one where a congenital abnormality associated with hydramnios led to fetal death immediately after cesarean section. This represented a rare opportunity to determine whether the tracer would concentrate in a specific organ or tissue. Since tritium was used as a tracer in all these cases, its detection and determination in solid material were relatively easy but an appropriate correction had to be made. These tracer analyses indi-

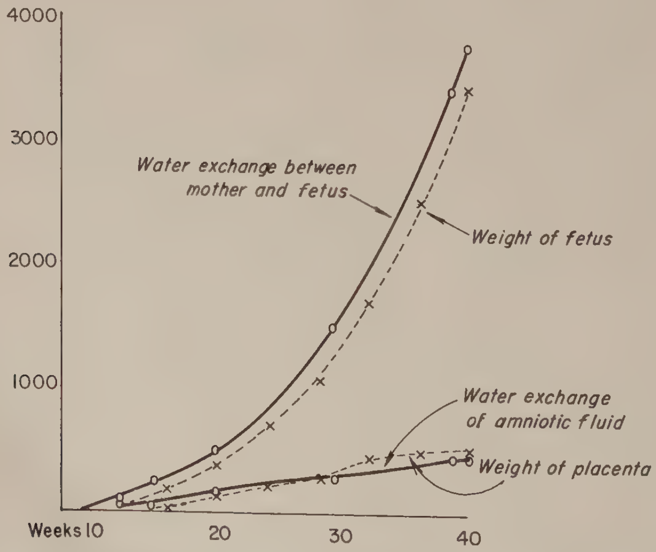

FIGURE 7. Comparison of water exchange and weights of products of gestation at various stages of pregnancy.

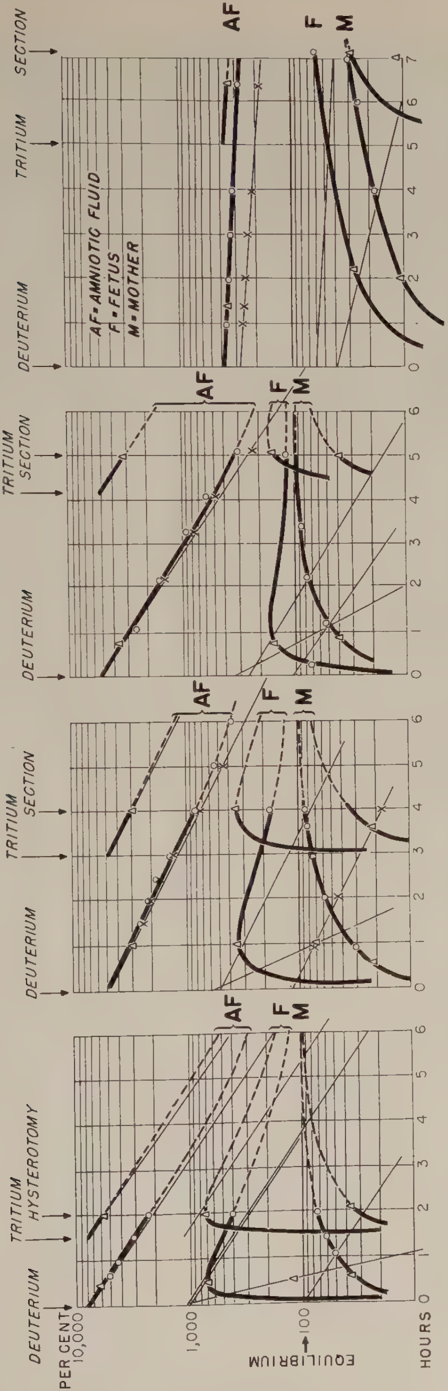

FIGURE 8. Comparison of tracer curves for amniotic fluid, mother, and fetus at 15, 30, and 40 weeks and under conditions of hydramnios.

cated that, within periods, as brief as 15 to 20 min., between 75 to 90 per cer equilibration takes place between fetal blood and all available tissues. Th analytic data on 4 of these experiments are given in TABLES 5 and 6. The origin assumption of rapid mixing in the fetal compartment (*in utero*) is thus reaso ably well established. Among other interesting findings was the presen of large amounts of tracer in the urine at 15 and 20 weeks gestation (compa FIGURE 6), which leads to the conclusion that, even at that early stage of pregnancy, the fetal kidneys function with reasonable efficiency. The only tissu that contained the tracer in concentrations many times higher than in co or fetal heart blood was Wharton's jelly. This leads one to suspect that th cord has the ability to concentrate the tracer in some fashion or, more likel that it constitutes a major pathway of exchange.

Throughout pregnancy water exchange rates seem to vary a great deal. Th six rates that constitute a theoretical minimum can vary despite their inte dependence, and it is therefore difficult to generalize. During the early par of pregnancy the quantity of water exchanged between mother and fetus an between mother and amniotic fluid is almost the same. During the remainde of gestation the mother-to-fetus rate rises rapidly at about the same rate a fetal growth, while the exchange of amniotic fluid water increases at abou the same pace as placental weight (FIGURE 7). Some time during the development of hydramnios an impairment of the maternal-fetal exchange take place, apparently without affecting the other exchanges. This produces picture superficially similar to that of the twelfth week of gestation. Unde conditions of hydramnios, the ratio of fetal weight to amniotic fluid volum also is similar to early pregnancies, but the transfer mechanisms are totall different, because weight and volume are more than ten times larger.

In order to provide the proper perspective for these exchange mechanisms i relation to the progressive aging of gestation, the combined graph in FIGURE was constructed. From left to right, the curves obtained at 15, 30, and 4 weeks and with hydramnios are compared. The picture is semischemati but all experimental points are taken from the original graphs previously presented. They are all referred to the same equilibrium value of 100, and th abscissa has the same time intervals.

The highest peaks are obtained for the smallest fetus. The maximal valu for the fetal tracer curve declines near term, producing a flatter curve wit relatively little change in the other compartments. Severe hydramnios—als at term—is associated with a time-activity curve strikingly different fro those of the normal. This difference is caused by a relative but sharp declin in the exchange between the maternal and fetal systems, and cannot be accounted for by the increased volume of the amniotic fluid alone.

Finally, a peculiar parallel exists between the magnitude of the exchang between mother and fetus as pregnancy progresses and the rise in fetal weight. A similar correlation can be demonstrated between the changes in placenta weight and the water exchange of the amniotic fluid.

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BLOOD FLOW OF THE GRAVID UTERUS*

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The gravid state imposes widespread changes upon the maternal organism These represent adaptive responses that permit the mother, under norma circumstances, to negotiate pregnancy without difficulty or harm. From teleological point of view, such gestational alterations are related to materna efforts to provide a proper environment for intrauterine growth and develop ment. In addition, they include maternal adaptations to fetal demands. Basi to any understanding of the physiological determinants of the mechanisms o reproduction is the consideration of the nature of the regional uterine circu lation. It is the purpose of this report to summarize briefly the present statu of pertinent information relative to changes in amount and quality of bloo flow in pregnancy.

Historically, while the concept of uterine ischemia long has been incriminate as the etiological factor underlying abnormalities in gestational course an growth, there have been few measured data to establish standards or evaluat deviations from it. An attempt to ascertain relevant facts in studies of th pregnant rabbit uterus was first made in 1932 by Sir Joseph Barcroft,[1] wh performed characteristically direct experiments that involved ligating al the tributaries of the inferior vena cava except the uterine veins. In thi animal preparation, all the blood returning via the vena cava of necessity had come through the uterine venous channels. A cannula was tied into on of the severed iliac veins and, at a signal, a bulldog clamp was switched from above the iliac vein cannula to the cava above the point of entry of the uterin veins. The iliac vein cannula outflow was measured over a given period o time. This was a crude measure of uterine vein flow, but it did indicate th magnitude of flow through the uterus. Barcroft further collected the venou blood from the venous cannula under oil, so that analyses could be done fo oxygen and carbon dioxide. Moreover, he collected simultaneous arteria samples that were analyzed also for the respiratory gases. When the uterin arteriovenous differences of oxygen and carbon dioxide were multiplied by the blood flow through the uterus per minute, Barcroft had an estimate of the pregnant uterus' oxygen consumption and carbon dioxide production. Additional uterine measurements were obtained in pregnant sheep, guinea pigs and in one instance, utilizing a cleverly designed fetal plethysmograph, in a human fetus of 12 weeks gestation.[2] By this technique, Greenfield measured the rate of umbilical blood flow by selective occlusion of the umbilical vein without altering the umbilical arterial flow. He related the initial decrease in the volume of the fetus to a measure of umbilical blood flow because its blood was rapped in the placenta. Simultaneous sampling of maternal arterial and terine venous, along with umbilical arterial and venous blood, was accomlished, and measurements were made of their respective content of the respiraory gases. The tolerable assumption was made that fetal oxygen consumption which was calculated) was equal to the total oxygen consumption of the terus. Uterine blood flow determined from this assumption is equal to the xygen consumption of the fetus divided by the maternal arteriovenous oxygen ifference across the uterus.

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This was the background of estimations of uterine blood flow in pregnancy experimental animals when, in 1950, our group at the Boston Lying-In Iospital sought to test the feasibility of adapting the Fick principle[3] to measureients of uterine blood flow in human pregnancy. At about the same time, . S. Assali, in Cincinnati, and J. C. McClure-Brown and N. Veall, in London, nd their co-workers concerned themselves with the same problem. The nglish group calculated blood flow from the disappearance curves of Na^{24} njected transabdominally into the placental intervillous space. The latter as located by scanning the abdomen with a Geiger counter for localized conentrations of radioactivity following a cubital vein injection. The thinking the Cincinnati group appeared to be comparable to our own. Both of us ere influenced by Kety's beautiful work wherein he adapted the Fick principle measurements of cerebral blood flow, utilizing 15 per cent nitrous oxide as e test substance.[4] The reports of McClure-Brown and Veall[5] and Assali *al.*[6] are now part of the record.

Our work and their reports are not without limitations and criticisms, hower. The use of radioisotopes in pregnancy, the critical assumption that the ncentration of radioactivity as detected by a Geiger counter identifies the tervillous space, and the significance of the mathematical interpretation of e slope of the disappearance curve have all been items for appraisal in evaluting the work of the London group. The Cincinnati team sampled uterine enous blood through a cardiac catheter that was introduced through a cubital ein and guided under fluoroscopy through the right sided chambers of the eart into the inferior vena cava to the level of entrance of the uterine vein. hey assumed that the uterus and its contents were at equilibrium following a nite period of inhalation. We elected to sample the uterine venous blood nder direct vision by catheterization of a venous tributary. We found further at, because the uterus and its contents, namely, fetus, placenta, and amniotic uid, were not homogenous (as was the brain in Kety's experience), it was ecessary to make a number of essential assumptions since total tissue saturaon did not occur. The remainder of this report is a discussion of our own perience in measurements of uterine blood flow in human pregnancy.[7, 8]

FIGURE 1 is modified from Kety and represents the physiological derivation the Fick equation applicable to any tissue. We reasoned that the method r estimating cerebral blood flow could be applied to the uterus if the amount nitrous oxide in the uterus at the end of the experiment could be deterined and if the concentration of nitrous oxide in arterial blood supplying d venous blood draining the uterus throughout the experiment could be

$$\text{Amount of test substance X left in organ during experiment} = \text{Amount of X brought to organ by arterial blood} - \text{Amount of X taken away from organ by venous blood}$$

Now:

$$\text{Amount of X brought to organ by arterial blood} = \text{Blood flow} \times (X)_{\text{Arterial}}$$

$$\text{Amount of X taken from organ by venous blood} = \text{Blood flow} \times (X)_{\text{Venous}}$$

So:

$$\text{Amount of X left in organ during experiment} = \text{Blood flow} \times \left[(X)_A - (X)_V \right]$$

Or:

$$\text{Blood flow} = \frac{\text{Amount of X left in organ during experiment}}{\left[(X)_A - (X)_V \right]}$$

FIGURE 1. Modified from Kety. Physiological derivation for determination of blood flow in any organ.

measured. Under such circumstances the general Fick equation toward its uterine application would be:

$$\text{Uterine blood flow} = \frac{\text{cc. } N_2O \text{ in uterus at end of experiment}}{(N_2O)_A - (N_2O)_V \text{ during experiment}}$$

The denominator, that is, the arteriovenous difference of nitrous oxide during the time of the experiment, can be determined by sampling arterial and venous blood simultaneously throughout the period of nitrous oxide administration to the mother and plotting the arterial and venous values so obtained against time. The integrated A-V nitrous oxide difference is represented by the area between the arterial and venous curves. We calculated the average arteriovenous nitrous oxide difference for each minute of the experiment as determined from the curves, and totaled these values to obtain the A-V nitrous oxide difference during the experiment.

The determination of the total uterine uptake of nitrous oxide or any other test substance in uterus, baby, placenta, and amniotic fluid (the numerator of the equation) has been the major problem. In the case of the brain, Kety found that at the end of 10 minutes the concentration of nitrous oxide in the brain reached equilibrium with the blood coming to it in the artery and departing in the vein. Thus, he was able to substitute blood concentration for brain content. Cerebral blood flow was expressed as cubic centimeter per hundred grams of brain. The pregnant uterus and its contents did not saturate equally and, even after periods of inhalation of nitrous oxide of up to 1 hour, there was no established equilibrium with maternal blood. Moreover, it was clear that we were not interested in blood flow equated against total uterine and intrauterine mass. If we were to express uterine blood flow as total flow per minute, it would be necessary to make certain assumptions and calculations in order to estimate the nitrous oxide content of the uterus and its contents. These involved the following considerations:

(1) The fetal and placental tissue are in nitrous oxide equilibrium with umbilical arterial blood. This assumption is supported by experiments in lower animals, where the fetal nitrous oxide content has been directly determined and compared with the nitrous oxide content of umbilical arterial blood (footnote, p. 1635, Metcalfe *et al.*[8]).

(2) The myometrium is in nitrous oxide equilibrium with maternal arterial lood at the end of 30 min. This equilibrium time has been found to be adeuate for myocardium and for brain. We have found that the solubility of itrous oxide in myometrium is identical with its solubility in blood. Hence, he maternal arterial concentration of nitrous oxide is used for the myometrial oncentration.

(3) The myometrium at term weighs 1 kg. This is an average figure taken om Reynolds.[9]

(4) The volume of amniotic fluid at term is 750 cc. This, too, is an average gure. However, it is relatively unimportant because of the low nitrous oxide ontent of amniotic fluid even after 30 min. of nitrous oxide inhalation by the nother. In actual experience, variation in amniotic fluid volume from 100 cc. o 1500 cc. was calculated to a uterine blood flow difference of only 70.0 cc./min.

With the assumptions listed above, we required certain data in order to make he final calculations. These could be obtained at cesarean section, and inluded (1) the weight of the infant, (2) the weight of the placenta, (3) the conentration of nitrous oxide in umbilical arterial blood and in umbilical venous lood, and (4) the concentration of nitrous oxide in amniotic fluid.

Our initial studies were confined to cases of elective repeat cesarean section n normal women between 38 to 40 weeks' gestational age. The technical

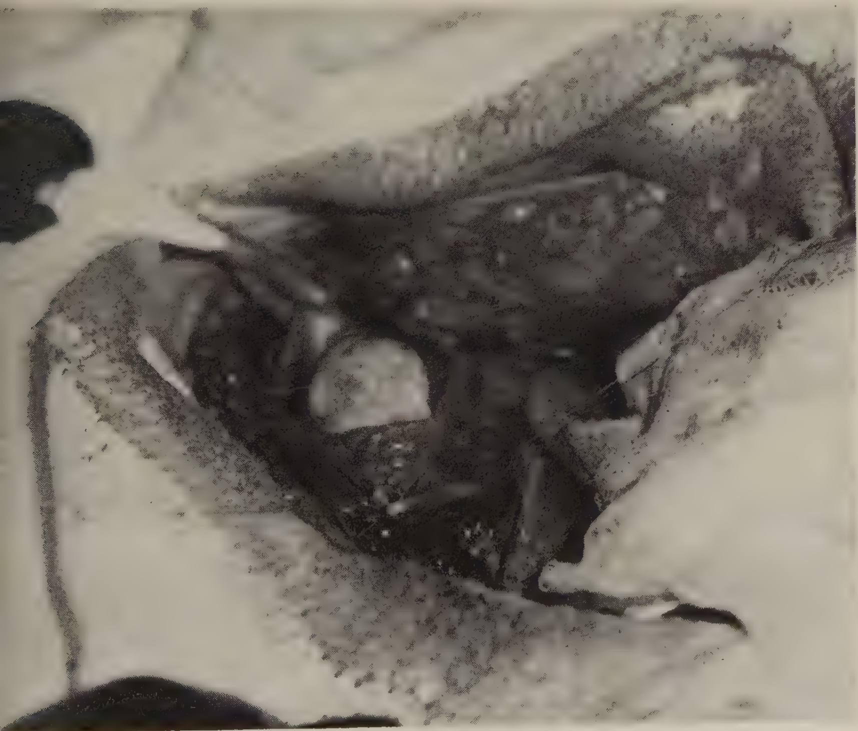

FIGURE 2. Isolated uterine venous tributary in spasm following dissection.

considerations have been summarized previously.[10] In brief, a tributary o the uterine venous pampiniform plexus is catheterized under direct vision. Th vasospastic characteristics of these vessels aid considerably in their isolatio (FIGURE 2) and catheterization (FIGURE 3). Following a 30-min. period o simultaneous arterial and venous sampling of the test substance (15 per cent

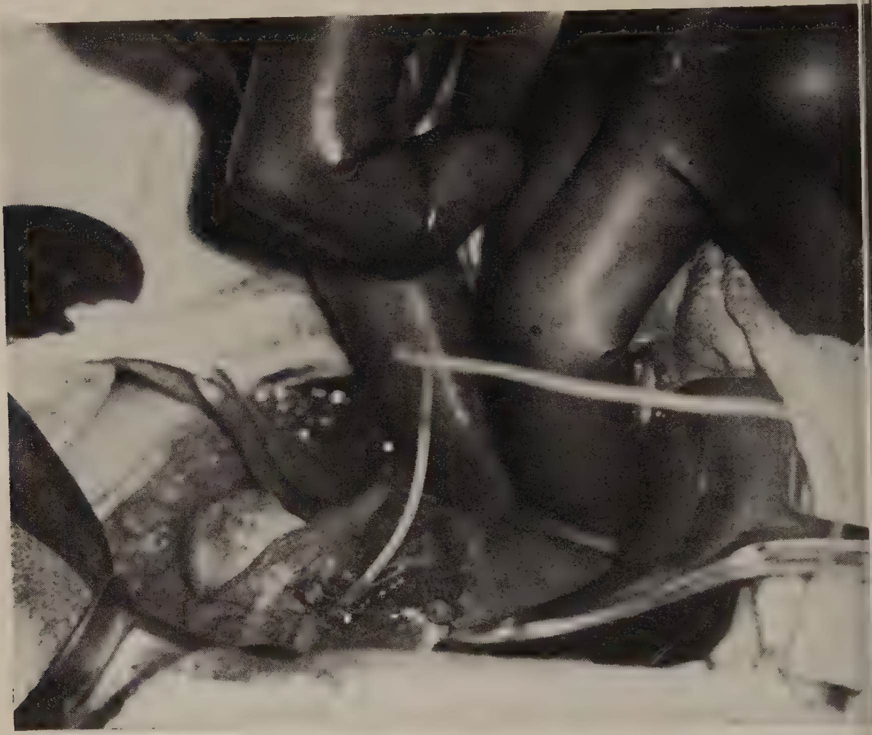

FIGURE 3. Incision of vein and direct catheterization with polyethylene tubing.

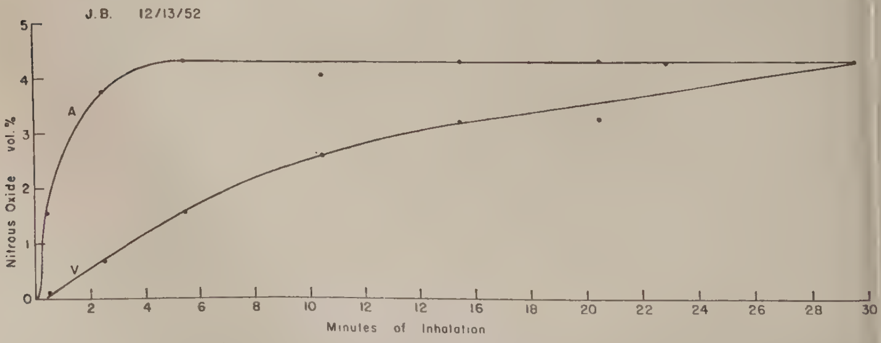

FIGURE 4. Arterial and venous curve obtained following period of nitrous oxide inhalation.

itrous oxide), we obtain a curve as seen in FIGURE 4. The uterus is then ncised through a lower uterine incision, and the fetus is delivered directly. A egment of umbilical cord is obtained between clamps and blood is rawn anaerobically from the umbilical artery and vein in heparinized syringes or nitrous oxide, oxygen, and carbon dioxide determinations. These are done n duplicate by the methods of Van Slyke and Neill[11] and Orcutt and Waters,[12] espectively.

FIGURE 5 summarizes the data obtained in a specific case while utilizing the ssumptions previously described. With the determined numerator equal o a value of 192, and the A-V area calculated to be 36, the uterine blood flow s determined to be 192/36 × 100 = 533 cc./min.

FIGURE 6 is a summation of fourteen studied cases. When this rate of uterine lood flow is considered with respect to the differences in oxygen and carbon ioxide content of uterine artery and vein, we obtain some idea of uterine xygen consumption, carbon dioxide production, and respiratory quotient. urthermore, if we make the admittedly incorrect assumption that the oxygen onsumption of the uterus is equal to the oxygen consumption of the fetus, we an calculate the rate of umbilical blood flow. In actuality, the uterine muscle ust consume some oxygen; hence, the figure obtained by dividing the uterine

$$\text{Uterine Blood Flow} = \frac{\text{cc. } N_2O \text{ in uterus at end of experiment}}{(N_2O)\text{ Artery} - (N_2O)\text{uterine Vein}} \qquad \text{(Equation 1)}$$

		Example - J.B. 12/13/52	
N_2O in amniotic fluid	= Vol. amniotic fluid x N_2O in amniotic fluid	750 cc. x 1.9 cc./100 cc.	= 14
plus			
N_2O in fetus and placenta	= Wt. fetus + placenta x N_2O in umbilical artery	3620 gms. x 3.4 cc./100 cc.	= 122
plus			
N_2O in myometrium	= Wt. myometrium x N_2O in maternal artery	1000 gms. x 5.6 cc./100 cc.	= 56
N_2O in uterus at end of experiment			192 cc.

FIGURE 5. Application to a specific case of the theory in the calculation of the numerator f the Fick equation.

	Average of 13 Women with Single Pregnancies	One Woman with Twin Pregnancy
Uterine Blood Flow cc./min.	490	1150
Uterine Oxygen Consumption cc./min.	25	48
Uterine CO_2 Production cc./min.	20	47
Uterine RQ	0.80	0.98

FIGURE 6. Summation of data obtained in fourteen cases of human term pregnancy.

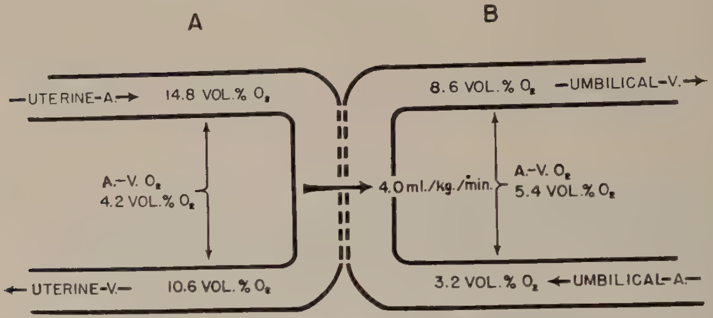

FIGURE 7. Arteriovenous oxygen difference of maternal (A) and fetal (B) blood at term.

oxygen consumption by the umbilical arteriovenous difference is bound to be on the high side.

The data thus far obtained suggest an order of magnitude of approximately 500 cc./min. allotted to the uterus at term. This is about half as much as the increase in maternal renal blood flow in pregnancy. The arteriovenous oxygen differences across the uterus and in the umbilical circulation are summarized in FIGURE 7. The evidence on the maternal side suggests an A-V difference across the placenta that is less than the average for the whole body. This is evidence in favor of the suggestion made about 15 years ago by Burwell and Strayhorn[13] that the placenta is an arteriovenous shunt. One of the cases studied involved a twin pregnancy. The data imply that twinning imposes twice the load on the maternal circulation. The average oxygen consumption per minute of the pregnant uterus at term is 24.5 cc., and the carbon dioxide consumption, similarly calculated, is 21.8 cc.—yielding an average uterine respiratory quotient of 0.91. The average uterine arteriovenous oxygen difference in the single pregnancies is equal to 4.8 volumes per cent. Previous calculation of the oxygen consumption of the human newborn[14] indicates it to be between 6 to 8 cc./kg./min. If we assume the myometrium to weigh 1 kg., and the placenta to weigh 550 gm., and assume that these tissues consume oxygen proportionately as does the fetus, the average oxygen consumption per kilogram of tissue per minute is 5.05 cc. This figure is of the proper order of magnitude when compared with animal observations.[15, 16] It is as if the same amount of oxygen is required to produce fetal tissue, gram for gram, regardless of the species involved.

Summary

Estimations of uterine blood flow have been determined in human pregnancy at term. The methodology is dependent upon a number of critical assumptions. These are supported by data obtained in experimental animals. At the same time, the arterial and venous maternal and fetal umbilical blood have been analyzed for the respiratory gases. The data provide an order of magnitude of about 500 cc./min. as representing the amount of maternal cardiac output diverted to the uterus; the uterine oxygen consumption is approximately 25 cc./min., while the fetus consumes approximately 5 cc./kg./min.

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Discussion

Hugh G. Grady (*Seton Hall College of Medicine and Dentistry, Jersey City, N. J.*): The papers included in this section serve to summarize in admirable fashion the complex and dynamic muscular, vascular, and fluid mechanisms utilized by the gravid uterus in fulfilling its destiny.

Reynolds' paper, based on his own fundamental investigations of the adaptation of the muscular uterus to its burgeoning content, is a masterly presentation of the work of many years. Böving's application of histological and physical methods to the analysis of implantation does much to make intelligible a mysterious but obviously not random process. The value of techniques that at least approximate the physiological is emphasized again by Ramsey's study of the uteroplacental circulation. Much the same can be said of Romney's investigation of human uterine blood flow, a problem of obvious complexity beset by limitations on the use of human subjects. To those who remember the concept of amniotic fluid as a sort of cesspool that functioned also as a cushion and finally as a flush box, Plentl's demonstration of the high rates of exchange on the fetal-maternal-amniotic axis is a challenge to rethinking and re-evaluating the significance of amniotic fluid.

Part VI. Uterine Contractions

INNERVATION OF THE HUMAN UTERUS*

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Although the innervation of the uterus in both humans and animals has been studied for several hundred years, only recently has the physiological correlation of our anatomical and histological observations been possible. This paper reviews the subject and adds further data wherever possible to the multitude of observations, both gross and microscopic, that have been made of this area, which involves so many conflicting views.

Extrinsic or Gross Nerve Supply

Early workers were hampered in their attempts to understand more adequately the nerve supply to the pelvic viscera. Although they recognized the presence of nerve elements in these organs, they were unable to distinguish types and cell structure. Among the early investigators were Wilissius in 1680,[1] de Graaf,[2] who in 1705 described the nerve supply to the ovaries and uterus, and Eustachius,[3] who went so far as to describe in detail the pelvic plexus and what later became known as ganglia. Most early investigations were little more than explorations. In the latter part of the Eighteenth Century and in the early Nineteenth, when better techniques had been worked out, descriptions by Hunter,[4] Tiedemann,[5] and others[6] began to establish the presence of a nerve pattern in the pelvis. It was actually Walter[7] who, in 1783, established the presence of ganglia in the region of the isthmus of the cervix. Other dissectors believed they could trace nerves directly into the uterus itself. As a result of the dissections of Haller,[8] Hunter,[4] and others,[9] the hypogastric, aortic, and spermatic plexuses were seen to be real entities. Following this, these plexuses were further elaborated into the various components of the sympathetic network in the pelvis.[5, 7, 10]

Although Lee[11] was the first to establish a true preaortic plexus and its connection with ganglia adjacent to the uterus, his ultimate proof, the microscopic, was lacking. This remained for others to establish.[12, 13]

Frankenhäuser, in his extensive study published in 1867,[13] attempted to correlate all the work of the previous investigators; in addition, he included his own gross and microscopic findings. He described a connection between the thoracic and the abdominal ganglia. Moreover, he agreed that there was a plexus in the preaortic area and described it, as others had already done.[5, 8, 11, 14-16] This was an error of dissection, according to Davis,[17] who was of the opinion that this so-called plexus was the aortic adventitia. However, the existence of a nerve plexus in the lumbar region is now well established,

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† Markle Scholar in Medical Science.

although whether it can properly be called preaortic is still a question. Davis,[17] on the basis of his own dissections, believes that the vagus nerve is connected with the "solar plexus," that is, the plexus and the abdominalaortic plexus, and other observers are of the same opinion.[5, 8, 13] Some have claimed a definite number of ganglia in the plexus, but most investigators agree that the number varies.[17, 18] The ganglia are for the most part paired and joined by a network of nerve bundles. Anastomoses occur with the lumbar sympathetics, which lie laterally on each side of the vertebral column. This abdomino-aortic-intermesenteric plexus terminates in the superior hypogastric plexus.

Much has been discovered that contributes toward the further understanding of the pelvic segment of the autonomic nervous system.[4, 5, 7, 10, 11, 13-33] The result has been variations of the original ideas of Lee[11] and Frankenhäuser,[13] and, more recently, the excellent work of Davis[17] and Labate.[18] The findings of all these workers may be summarized as follows.

The pelvic autonomic system can be divided into the superior hypogastric plexus—the presacral[11] plexus and the uterinus magnus[13]—the middle hypogastric plexus, and the inferior hypogastric plexus. The superior hypogastric plexus begins just below the inferior mesenteric artery. It is composed of from one[27, 34] to three[35-37] intercommunicating nerve bundles[38] connected with the inferior mesenteric ganglia,[17, 18, 28, 32] and no ganglia is an integral part of the plexus. On the other hand, there are those[29] who believe that the greatest number of nerves in this plexus arise from the lumbar ganglia. There is no doubt that the intermesenteric nerves receive branches from the lumbar sympathetic ganglia.[18-29]

The superior hypogastric plexus continues into the middle hypogastric plexus.[13, 17] Some consider it to be a segment of the superior hypogastric plexus, rather than a separate entity.[18] The "presacral nerves" spread out into a latticework at the level of the first sacral[28] vertebra, with connecting rami to the last of the lumbar ganglia.[17] The greater part of the superior-middle hypogastric plexus may be found to the left of the midline.[23] At the first sacral vertebra, this plexus divides into several branches, which go to the right and left sides of the pelvis. These form the beginning of the right and left inferior hypogastric plexus.[13, 17, 32, 38] The inferior hypogastric plexus, which is the divided continuation of the middle hypogastric plexus,[17] the superior hypogastric plexus,[18] the presacral nerve,[13, 18] and the uterinus magnus,[13] is composed of several parallel nerves on each side. This group of nerves descends the pelvis in a position posterior to the common iliac artery and anterior to the sacral plexus, curves laterally and, finally, enters the sacrouterine fold or ligaments.[11, 13, 17, 18] The medial section of the primary division of the sacral nerves [(S_2 S_3)[39] (S_2 S_3 S_4)[13] (S_3 S_4)[40] (S_1 S_2 S_3 S_4 ?S_5)[38] (S_2 S_3 S_4 ?S_5)[17]] sends fibers (nervi erigentes) that enter the pelvic plexus in sacrouterine folds.[13, 17, 18, 41] The plexus now appears to contain both sympathetic (inferior hypogastric plexus) and parasympathetic (nervi erigentes) components. The sensory components, which are visceral for the most part, are found in the nervi erigentes; however, if one takes into account the amount of spinal anesthetic necessary to eliminate uterine sensation, one must assume that there

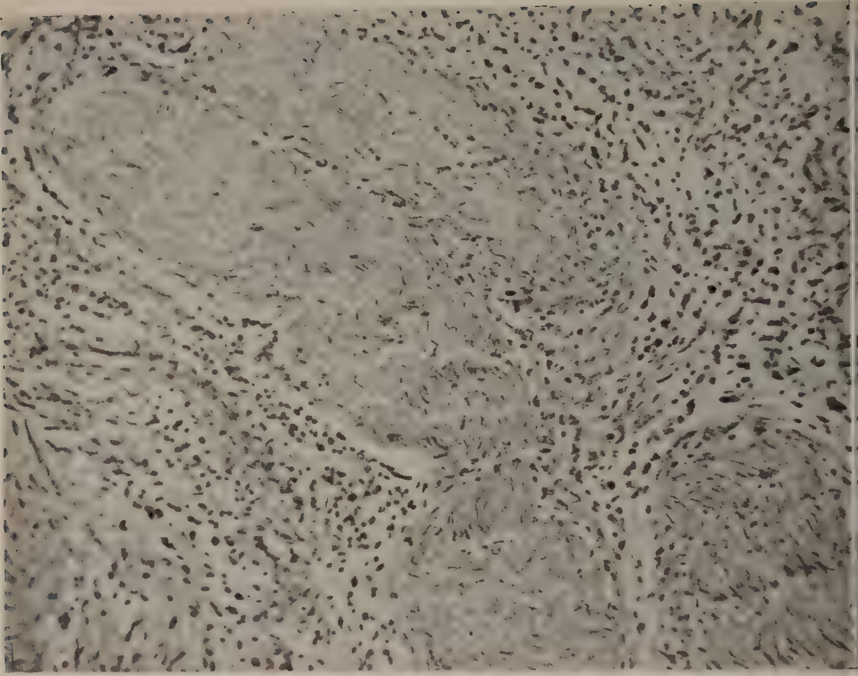

FIGURE 1. Photograph of nerve bundles in the isthmus of the cervix. The bundles contain both myelinated and unmyelinated fibers. ×250.

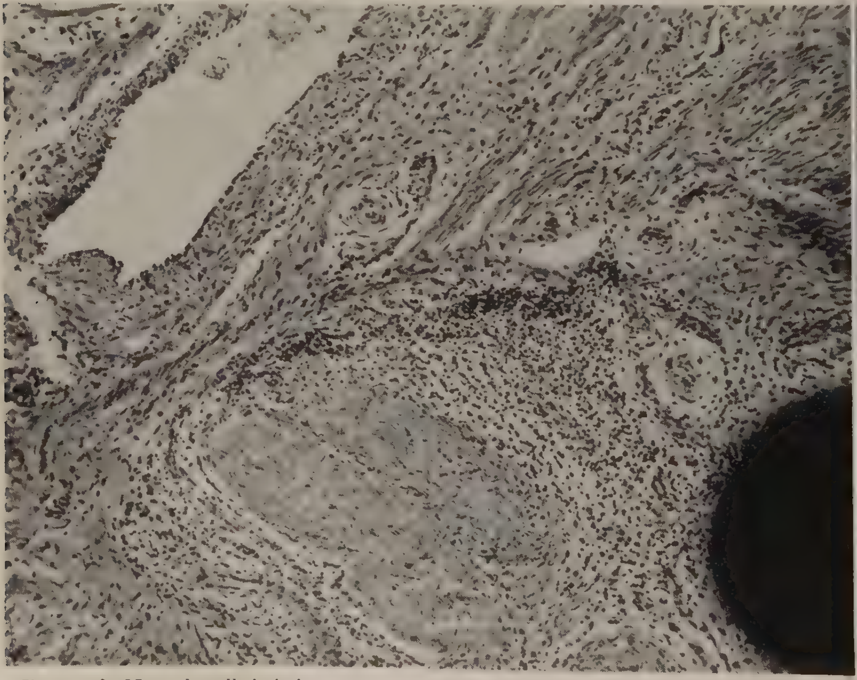

FIGURE 2. Nerve bundle in isthmus of cervix with two lamellar Dogiel-Krause type endings ×150.

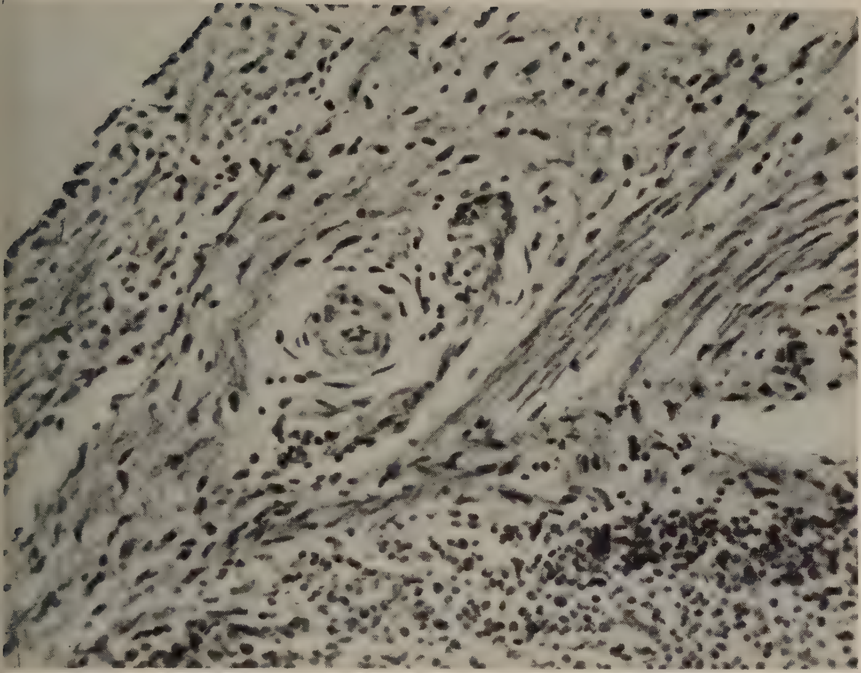

FIGURE 3. Photomicrograph of two Dogiel-Krause type lamellar endings in the isthmus portion of the human uterus. Note one is cut in longitudinal section and the other in cross section. ×240.

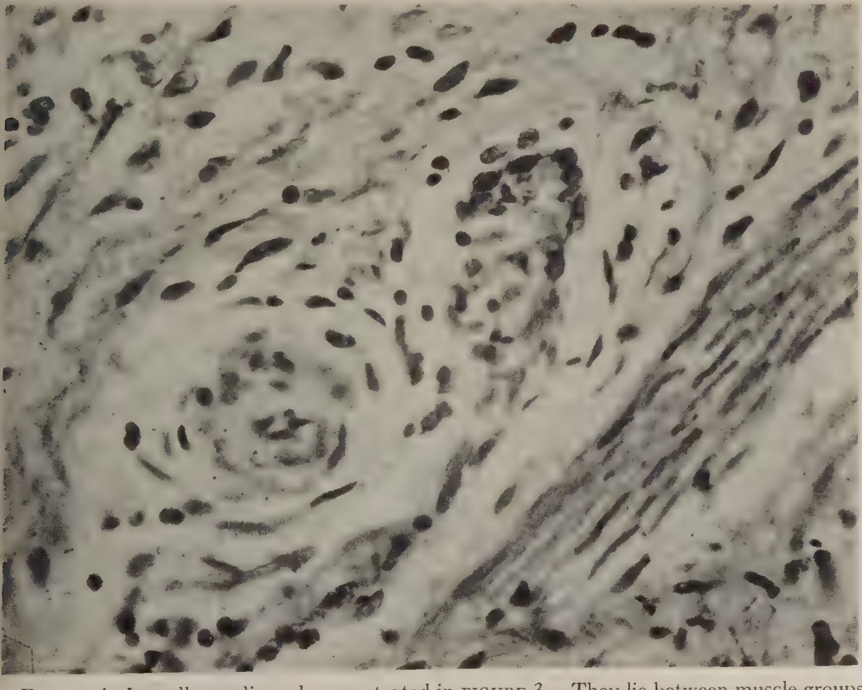

FIGURE 4. Lamellar endings deomonstrated in FIGURE 3. They lie between muscle groups adjacent to nerve bundles and frequently beside arteries. Note their lamellar structure and the modified epithelial cells in their structure. ×700.

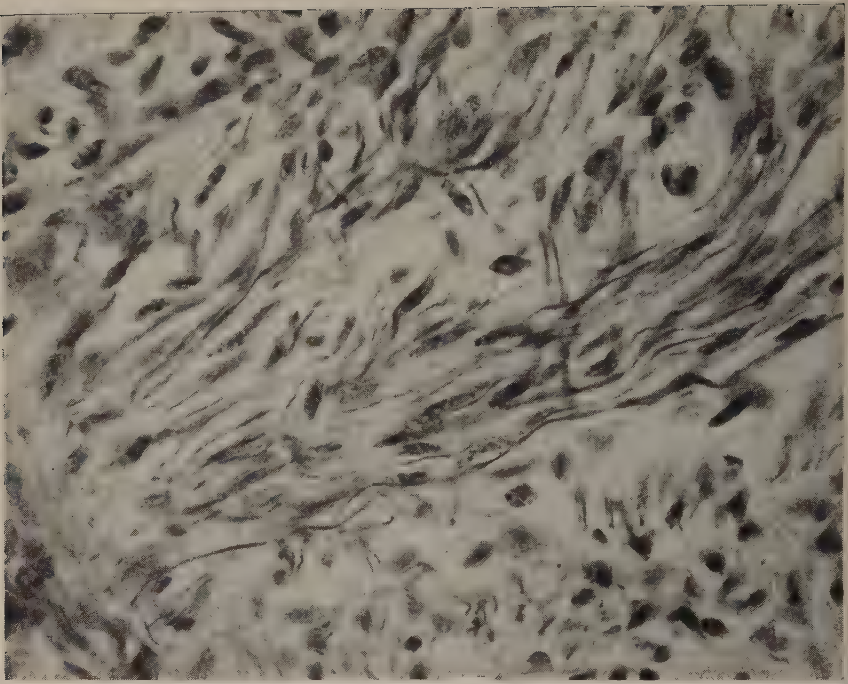

FIGURE 5. Nerve plexus among muscle cells in the lower portion of the corpus of the uterus. ×1050.

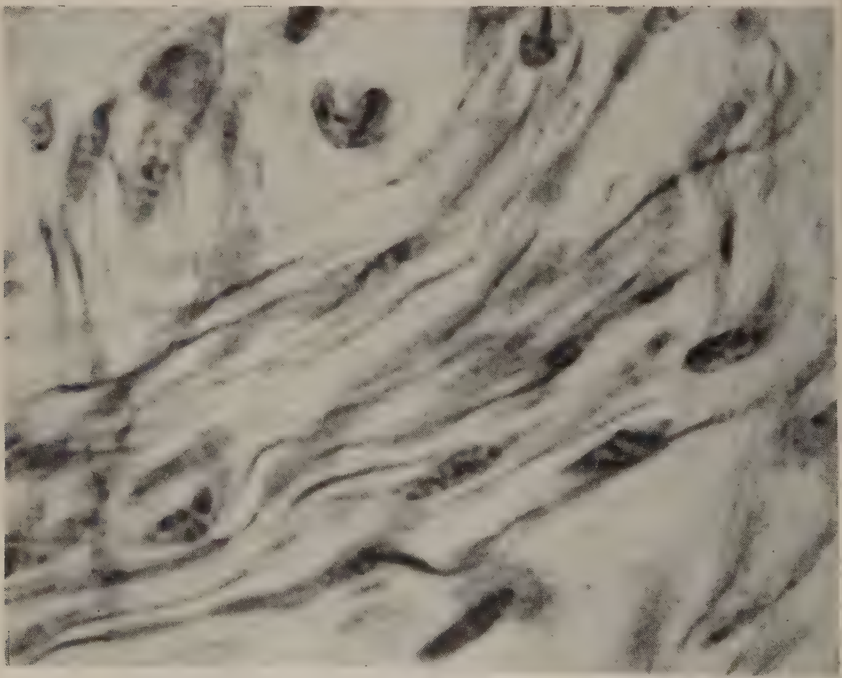

FIGURE 6. A higher power photomicrograph of FIGURE 5. There is frequent branching of fibers prior to their termination. Approximately ×2000.

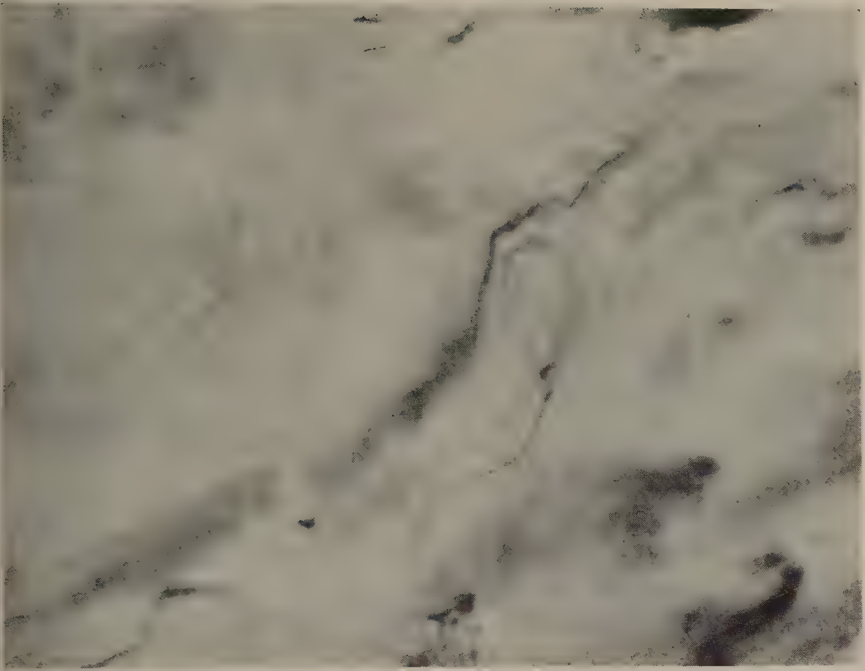

FIGURE 7. Nerve ending in contact with the wall of a muscle cell. Focus is difficult due to the thickness and magnification. ×2000.

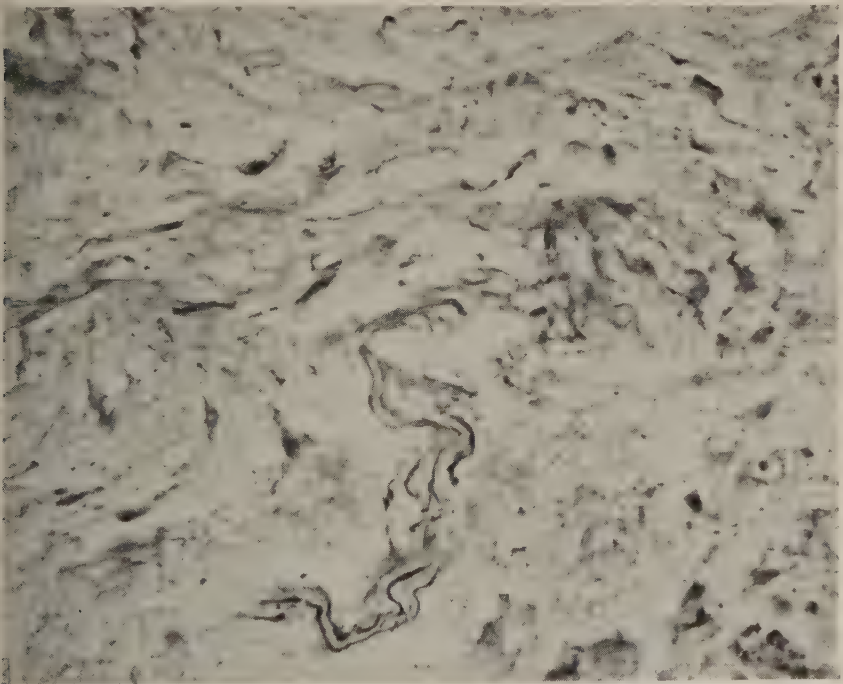

FIGURE 8. Nerves entering the adventitia of blood vessel in the corpus of the human uterus. ×900.

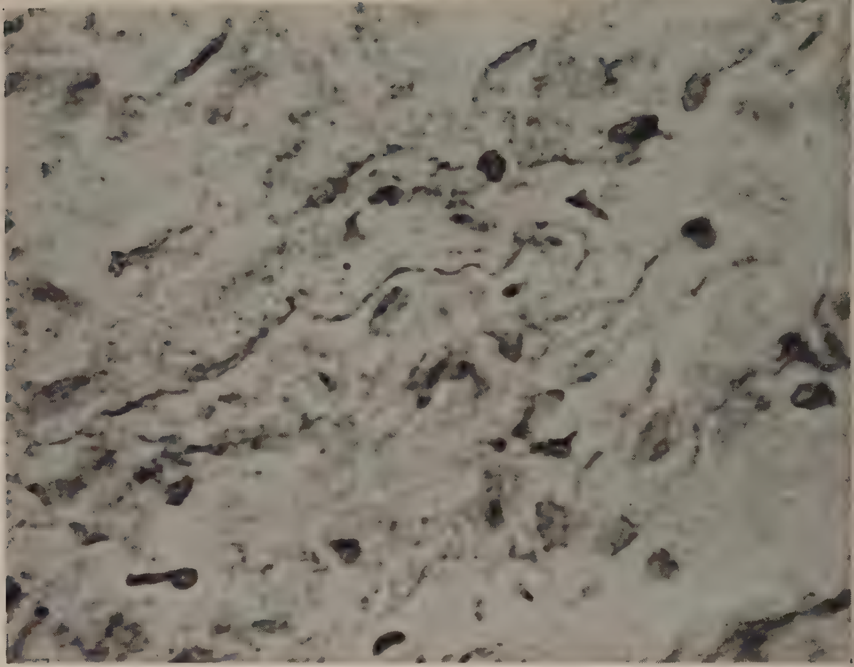

FIGURE 9. Nerve fiber in the cervix. Few nerves were seen in the tunica propria of the cervix-pars vaginalis. Only occasional fiber entered the skin. ×900.

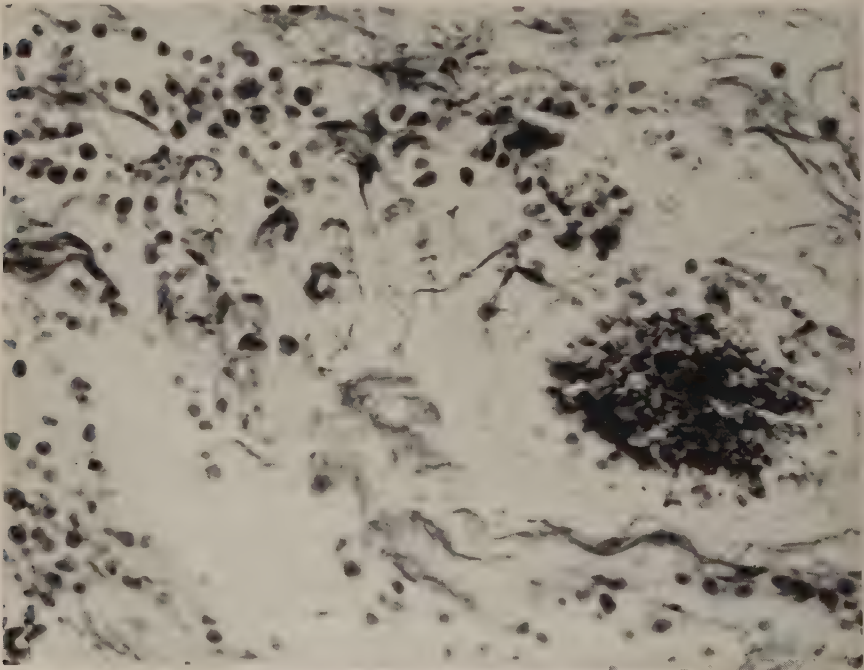

FIGURE 10. Section through the endometrium demonstrating free nerve endings in the stroma. The large black mass is the secretion within a gland. ×900.

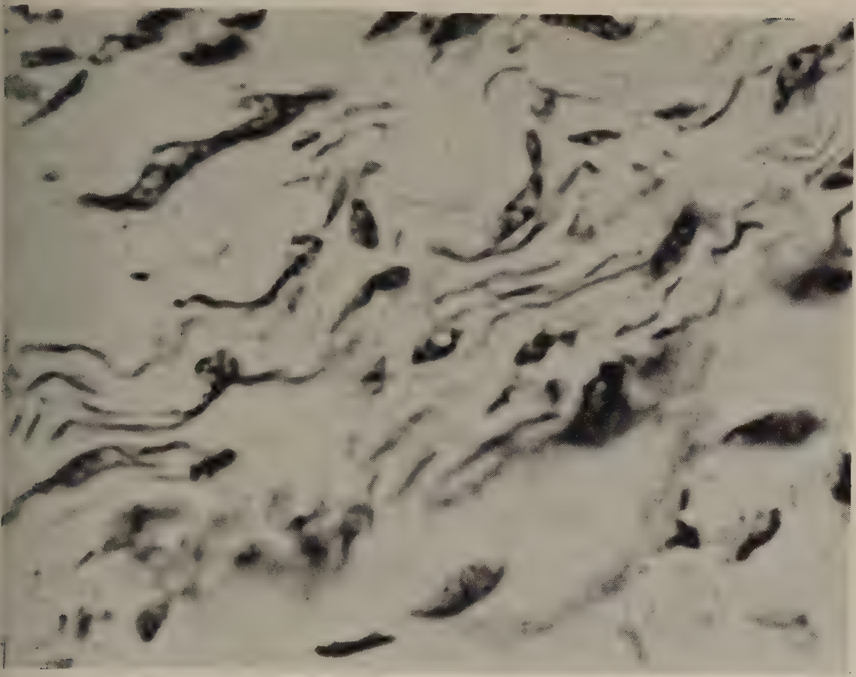

FIGURE 11. View demonstrating free nerve endings within the stroma of the endometrium. ×2000.

re a fair number of sensory fibers in the sympathetic component. The common iliac nerves originate separately from the superior hypogastric plexus and lescend on the surface of the artery and vein, one part joining the femoral ing, the remainder following the internal iliac, and finally rejoining the pelvic lexus.

On either side of the uterus in the base of the broad ligament is the large lexus described by Lee;[11] and Frankenhäuser,[13] namely, the so-called hypo- astric ganglion.[28, 30, 32] The plexus actually consists of many various-sized anglia and nerve ramifications, as well as branches of the joint inferior hypo- astric plexus and the nervi erigentes. It lies parallel to the lateral pelvic wall, ts lateral surface superficial to the internal iliac and its branches; the ureter ccupies a position superficial to the plexus.[17] The middle vesical artery perfo- ates and supplies the plexus,[17] its medial branches supplying the rectal stalk. Vhether any specific component of the plexus supplies one area more than nother is unknown.[17, 32, 42] As early as the time of Lee[11] and Frankenhäuser[13] was recognized that a greater part of the plexus terminates in large branches hat enter the uterus in the region of the internal os, while another smaller part f the plexus supplies the vagina and the bladder. The branches of the plexus hat supply the uterus enter the isthmus area primarily through the sacrouterine old or ligament.[11, 13, 17, 18, 41] In the isthmus just outside the entrance to the uterus, ascending rami pass out into the broad ligament; they enter the body of the uterus at higher levels besides supplying the uterine tube.[13] According to Davis,[17] part of the inferior hypogastric plexus passes directly to the uterus without involvement in the pelvic plexus.

Microscopic

The presence of ganglia in the pelvic plexus is now an established fact,[11, 13, 16, 17, 43-47] although it was once questioned.[15, 28, 30, 48-53] The extensive distribution of ganglia in this plexus has been apparent in my own studies. I found ganglia not only in close proximity to the uterine arteries and the ureters but also in the adventitia of the bladder and vagina, as well as in the vesico-vaginal septum. The nerve bundles entering the ganglia contain both myelinated and unmyelinated elements. The myelinated fibers vary considerably in size. The majority of cells in the ganglia are multipolar, the minority are the cogwheel and comet types. Pacinian corpuscles can be found within the tissues and are often observed within nerve bundles, especially within those in the lower divisions of the plexus. I also found that motor and sensory fibers cannot be discerned morphologically.[42]

Intrinsic Innervation

The investigation of the termination of the nerves of the pelvic plexus within the uterus depends on adequate techniques. These were not available until the early 1830s when, in Müller's laboratory in Berlin, Remak[12a] first described nerve fibers as a true entity both connected with, and an integral part of, a neuron. Then, in 1840, after microscopic study of various tissues of both animals and humans, he reported the presence of nerve fibers in the uterus.[12b] Doubts concerning Remak's original thesis were voiced by such men as Valentin,[14] Bidder and Volkmann,[54] Bischoff, as quoted by Henle,[40] and Henle[40] himself, Wagner, as quoted by Valentin,[14] and even, for a time, by Müller.[55] As a result, it was not until 1851, when Kilian[48] accepted Remak's "neuron-axencylinder" concept and demonstrated the presence of nerve fibers in the uterus, that their presence became an accepted fact. Once this had been established, the types and endings came under discussion,[11, 15, 56, 57] and to some degree this latter question is still an issue. It had once been thought that the nerves increased during pregnancy. However, as early as 1851[48] this concept was thoroughly refuted.

The question of whether there are myelinated[58] or unmyelinated nerves, or both, in the muscularis was resolved only recently. In 1867 Frankenhäuser, repeating the work of Kilian,[48, 59] described both types of fibers as present,[17, 60-63] although even in the early part of this century[64] some observers still believed that only one type of fiber was present. The presence of a plexus in the muscularis has been indicated.[42, 63, 65, 66] Moreover, most investigators agree that this muscularis has a rich nerve supply and that the nerve terminations in the smooth muscle endings are: unmyelinated fibers in contact with the smooth muscle sarcoplasm;[13, 42] a branching plexus ending in fine fibers within the sarcoplasm;[58, 63, 67-71] cone-shaped nerve endings on muscle cells;[16, 72] free endings among muscle fibers with terminal enlargements;[73, 74] a reticulum of neurofibrils forming a syncytium with buttonlike endings on smooth muscle cells;[34] and those among and alongside the smooth muscle cells.[17]

In my own researches with human material, in which I employed both Bielschowsky's method[75] and Davenport's modification of the Bodian method,[76] I found both myelinated and unmyelinated nerves within the uterus. These nerves enter, as previously described,[13, 17, 62, 77] along the blood vessels, the richest supply lying in the isthmic portion of the uterus. The fibers following the blood vessels gradually diminish in number in the direction of the fundus, where the sparsest distribution occurs. The fibers run parallel to muscle bundles, and the nerves frequently branch to form a syncytium before terminating on the sarcoplasm as small free nerve endings. Occasional free endings were also observed between the muscles; these were most numerous in the cervical area, but whether they were sensory free endings was not clearly determined.

The blood vessels of the uterus have been found to receive a rich supply of primarily unmyelinated fibers.[13, 16, 17, 34, 58, 63, 65, 78, 79] These fibers terminate among the muscle fibers of the vessels and adventitia as many free endings.[17, 63, 80] Keiffer believes that there may be even a small ganglion cell in this area.[79] Nerve fibers have been observed at the origin of the basal and spiral arteries of the endometrium.[63] How these fibers terminate is unknown.[63]

In my own studies with human material I observed many unmyelinated nerve endings among the smooth muscle fibers of the blood vessels, even at the origin of the spiral and basolar arteries of the endometrium. The endings appeared to be free and to lie among the muscle fibers of the vessels. No specialized ganglion cells were noted either in ganglia or as isolated individual cells.

The question of whether there are ganglion cells in the uterus has been studied for more than one hundred years. Many of the early workers believed they were present in one form or another.[17, 39, 60, 69, 73 75, 81-86] However, they were refuted by other observers.[11-13, 16, 20, 42, 47-49, 58, 63, 68, 70, 72, 74, 77, 78, 87-102] As recently as the 1930s Keiffer's thorough investigation[80] revealed what he thought were ganglion cells in the adventitia of some veins, and Davis[17] believed he had observed a modified ganglion cell in the region of the cervix, which was extramuscular but within uterine substance. My own study of the human uterus did not demonstrate ganglion cells within the muscularis of the uterus.

The presence of Vater-Pacinian corpuscles extrinsic to the uterus is well established.[77] On the other hand, although it is a matter of conjecture whether there are modified Dogiel-Krause corpuscles ending within the uterus,[16] some workers[34, 65, 80] believe there are. For example, in 1943 Hirsch and Martin[103] described a Dogiel-Krause type of corpuscle they had observed between large bundles of muscle in the human. I myself observed small lamellar endings similar in appearance to those described by Hirsch and Martin, which were situated between large bundles of muscle and adjacent to arteries. These endings appeared most frequently in the region of the endocervix. None were observed in the cervix, but there were a few in the corpus and fundus. They can also be found in the broad ligament along with Vater-Pacinian corpuscles as well as at the juncture of the uterine arteries with the uterus.

The innervation of the cervix as a separate entity has escaped most observers, although Davis[17] describes an extensive submucosal plexus, and Keiffer[30] goes so far as to describe corpuscles of the Vater-Pacinian type. My own study showed occasional free endings entering a papilla of the stratified squamous epithelium of the pars vaginalis. Beneath the epithelium, I found a rich plexus supplying the blood vessels of the cervix. The endocervix was observed to contain a rich plexus of free endings, which was most pronounced in the region of the internal *os*. The endocervix and the isthmic portion of the uterus in the nonpregnant state both contained the highest number of nerves and blood vessels of any part of the uterus. The presence here of a lamellar type of corpuscle has already been noted.

It was demonstrated by some of the early workers[13, 48] that nerves pass through the myometrium and enter the endometrium. A plexus involving the submucosal region with penetrating fibers is described by many,[16, 62, 65, 74, 81, 87, 104] although others disagree.[17, 72, 73] The excellent work of State and Hirsch in 1941[63] demonstrated that there are nerves in the basal third of the endometrium that have branches terminating in the stroma, in the basolar arterioles, and at the origin of the spiral arterioles. In that study the fibers in the endometrium were found to be unmyelinated. In my study an extrinsic plexus of myelinated and unmyelinated nerves was observed in the myometrium underlying the endometrium, the nerve elements following the vessels into the endometrium. The terminations were seen to be free endings in the stroma and in the adventitia of the basolar and spiral arteries at their origin. The outer two thirds of the endometrium was devoid of nerves.

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COMPARATIVE STUDY OF UTERINE CONTRACTILITY, URINARY ESTROGEN-PREGNANEDIOL LEVELS, AND MYOMETRIAL DISAPPEARANCE TIME OF RADIOACTIVE SODIUM IN FULL-TERM PREGNANCY AND PREMATURE DELIVERY

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Introduction

An investigation was begun in this department in 1951 to determine the possible existence of a relationship between urinary steroids and uterine contractility during normal pregnancy. Observations were made at two-week intervals on sixteen normal patients from the twentieth to the fortieth week of pregnancy. When the *mean* urinary estrogen and pregnanediol values were compared with uterine contractility during the second half of pregnancy, an abrupt rise in urinary estrogen and uterine contractility was seen to have occurred on or about the thirty-second week of gestation. The rise noted in urinary pregnanediol excretion was not as marked as that of urinary estrogen excretion.[1]

A second study was begun in 1953 to compare sixteen patients who delivered prematurely with the previously studied normal group. The majority of these patients who delivered prematurely showed a reduced urinary excretion of estrogen and excessive uterine contractility many weeks before the onset of labor. When prematurity was complicated by disease processes of a vascular nature, such as pre-eclampsia, essential hypertension, and renal disease, a reduction of both urinary estrogen and pregnanediol occurred in conjunction with uterine hypermotility. These results suggested that low urinary steroids and excessive uterine contractility bore some relationship to premature deivery. On the assumption that low urinary steroid levels and uterine hypermotility were not related as cause and effect, but were manifestations of another aberration, possibly a reduced uterine circulation, a further study was done in 1955 to compare the disappearance time of $Na^{22}Cl$ from the myometrium in normal full-term pregnancy to premature delivery. Results of this preliminary nvestigation utilizing the intramyometrial injection of radioactive sodium revealed an increased disappearance time of $Na^{22}Cl$ in eight patients who deivered prematurely, in comparison with eight normal patients who delivered at term.[2]

This report deals with these and further observations comparing uterine contractility, urinary steroids, and myometrial disappearance time of radioactive sodium in normal and abnormal pregnancy.[3]

Materials and Methods

All of the patients in these studies were delivered through the Obstetrical and Gynecological Service of the University of Colorado School of Medicine. Twenty-four-hour urine specimens were collected under butanol and stored.

Pregnanediol was determined gravimetrically by a modification of the Somme ville-Marrian and the Astwood-Jones methods.[4] Urinary estrogenic steroi were assayed colorimetrically by a modification of Engel's procedure.[5 Uterine activity was measured by the Reynolds tocodynamometer.[7] My metrial disappearance time of radioactive sodium was calculated by a metho similar to that described by Morris *et al.*[8]

Observations

Full-term pregnancy. From the twentieth to the thirty-second week normal pregnancy the uterus was relatively quiescent, showing no recordab activity or occasionally 1 or 2 contractions per hour. From the thirty-secor week until term, uterine activity increased to 4 or 5 contractions per hou As a parallel finding, from the twentieth to the thirty-second week of pre nancy the urinary estrogen levels increased slowly from 3 to 7 mg. per da while, from the thirty-second week to term, urinary estrogen excretion ro more rapidly from 7 to 20 mg. per day. The rise in urinary pregnanediol fro 20 to 50 mg. per day throughout the latter half of pregnancy was gradual ar more variable than the urinary excretion of estrogens (FIGURE 1).

Premature delivery. Sixteen patients who delivered prematurely had uteri contractility measurements and urinary estrogen-pregnanediol determinatio done at two-week intervals from the twentieth week of pregnancy to deliver Forty per cent of these patients delivered spontaneously for reasons unknow Sixty per cent had an associated complication of pregnancy. When compare to the normal group, all patients who delivered prematurely showed a trend c

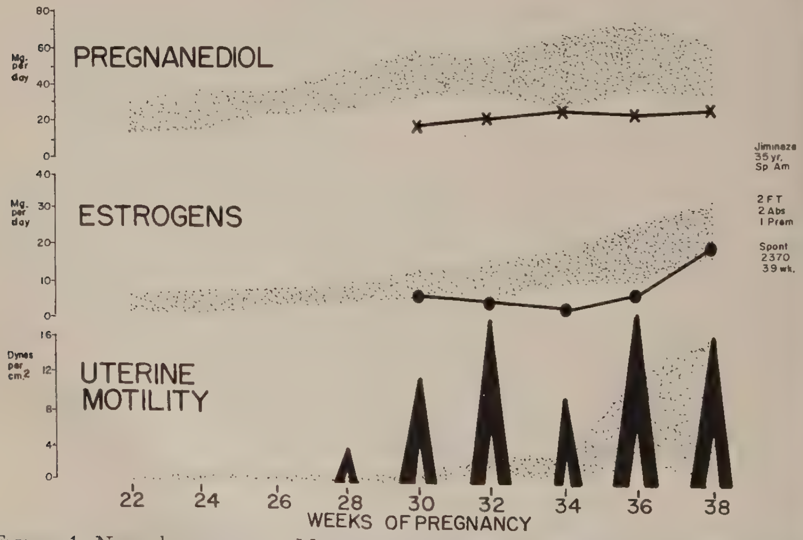

FIGURE 1. Normal pregnancy. Mean values of urinary pregnanediol and estrogen base on 10 normal full-term pregnancies. Uterine work depicted at 50 and 75 percentiles. Not the rapidly increasing values near the thirty-second week of gestation.

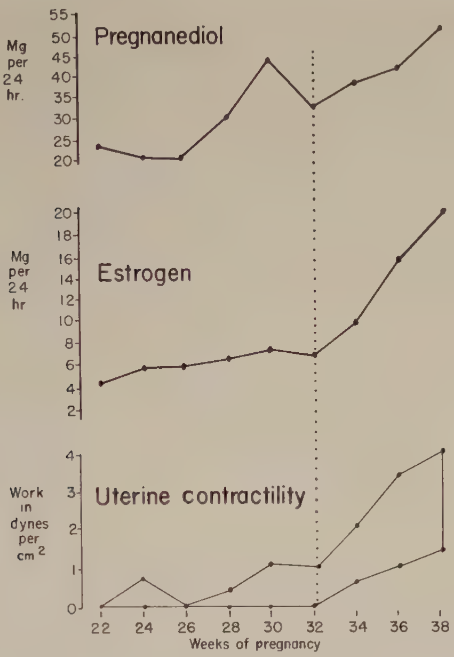

FIGURE 2. Pyelonephritis. This patient with toxemia showed low levels of both urinary teroids, as well as an excessive uterine contractility.

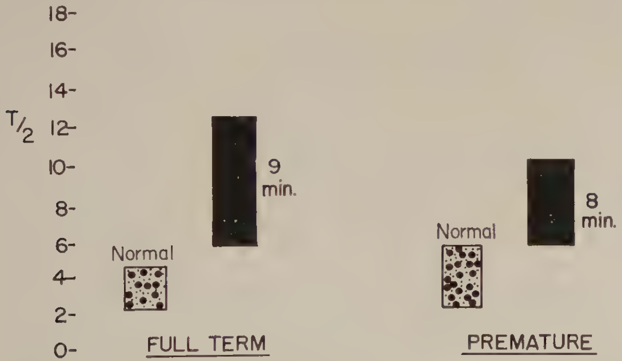

FIGURE 3. Uterine circulation in complicated full-term pregnancy. The disappearance ime of $Na^{22}Cl$ (T/2) from the myometrium was 9.0 min. In complicated premature de-very, the disappearance times were similarly increased to double the normal values.

ow urinary estrogen excretion and excessive uterine motility from as early as he twenty-sixth week of pregnancy to delivery. Patients in the premature roup with toxemia of pregnancy had low levels of both urinary estrogen and regnanediol. This is exemplified in one patient who developed pyelonephritis uring pregnancy (FIGURE 2).

Myometrial disappearance time of Na^{22}Cl. The disappearance of radioacti sodium from the myometrium, or "time to half value," from the twentieth the fortieth weeks of normal pregnancy in 64 patients was 4.0 minutes. Twent five patients who delivered prematurely had radioactive sodium disappearan times measured during the last half of pregnancy. In 13 of these patients wh had uncomplicated spontaneous premature labor, the mean disappearan time was 3.5 minutes. This figure was essentially the same as that found the normal group. When premature labor in 12 patients was complicated b twins, toxemia, bleeding, or infection, this half-time value increased to 8 minutes (FIGURE 3).

Discussion

It is important to emphasize the well-known fact that certain limitations ar imposed by the methods employed in this study. It is our opinion, howeve that external tocography, urinary steroid determinations, and myometri disappearance times are sufficiently accurate to provide useful informatio and clues for future investigation.

These studies were designed to investigate some aspects of the physiolog of normal pregnancy and to compare them to similar studies made of patient who delivered prematurely. From our observations of normal patients, it woul appear that when placental hormones (as reflected by urinary estrogen an pregnanediol levels) are being excreted in rapidly increasing amounts after th thirty-second week of pregnancy, spontaneous uterine contractility also in creases rapidly. From observations on patients who deliver prematurely, lov urinary estrogen levels (and low urinary pregnanediol in toxemia) were asso ciated with excessive uterine contractility. It was our impression that both c these defects, low urinary steroids and excessive uterine contractility, were no related as cause and effect, but might both be secondary to another aberration

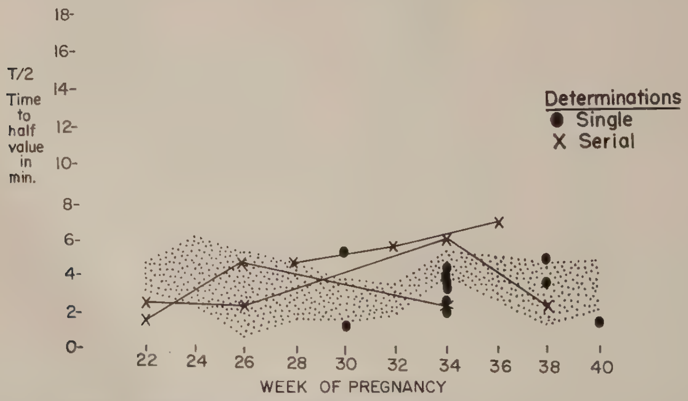

FIGURE 4. Uterine circulation in uncomplicated premature delivery. The disappearance time of radioactive sodium from the myometrium was the same as that observed in the normal group.

possibly a reduced uterine circulation. When this concept was tested, using the intramyometrial injection of radioactive sodium, the results were unexpected, for they differed from the values obtained in the preliminary study. Furthermore, in five uncomplicated patients who delivered prematurely and who had serial determinations done at four-week intervals, no change from the normal myometrial clearance times could be demonstrated (FIGURE 4).

These findings were contrary to our original concept that a defect in myometrial circulation might be the underlying mechanism in spontaneous premature labor of undetermined cause. However, this study did reveal that when pregnancy of any gestational length was complicated by toxemia, hemorrhage, infection, or twins, the myometrial disappearance time of radioactive sodium was increased to twice the normal value. If the disappearance time of radioactive sodium from the myometrium is a reflection of uterine blood flow, as previous investigators have stated, then these observations suggest a diminished uterine blood flow in complicated pregnancy of any gestational length as compared to normal gestation. Although this hypothesis is an attractive one, interpretation of these results in terms of blood flow remains unproved.

Summary

Patients who deliver prematurely show a reduction in urinary estrogen or pregnanediol excretion or both, and have an excessive uterine contractility when compared to normal patients. When pregnancy of any gestational length is complicated by toxemia, hemorrhage, infection, or twins, there is a reduction in the myometrial clearance of radioactive sodium. It is suggested that low placental hormone secretion and excessive uterine contractility might be related to a reduction in uterine circulation, especially in cases of toxemia of pregnancy.

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FUNCTION AND REGULATION OF THE MYOMETRIUM

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Students of gestation who attempt to explain the maintenance and termination of pregnancy, the mechanism of parturition, and the disorders of these processes must not neglect the embarrassing fact that human twins can be born two months apart from the same bicornate uterus (FIGURE 1). This simpl and direct clinical demonstration that a *local* factor in the uterus determine the maintenance and termination of pregnancy contradicts all current hypotheses concerning the regulation of myometrial function. The best we can do in view of this observation is to reconsider all available data on the subject, selec those that are compatible with this peculiar phenomenon, and propose a new theory, to be tested experimentally.

I propose here a brief review, in historical order, of the data that may give u a clue to the explanation of this striking clinical observation. I shall then proceed to propose a general theory of the endocrine control of the myometrium in pregnancy, based on observations pointing to a *local* effect of placental progesterone. Finally I shall discuss the delivery process.

In the year 1900, Gustav Born, the great embryologist of Breslau, lay dying. To his bedside he summoned his former student Ludwig Fraenkel and lef him an intellectual bequest. The legacy was the notion that the mysteriou yellow body, the corpus luteum of the ovary, is an organ of internal secretion responsible for the protection and development of the early embryo. This brilliant prediction led to many startling discoveries and dilemmas.

Fraenkel (1910) removed the corpora lutea from rabbit ovaries after ovulation and fertilization had occurred, by burning them out with a fine cautery. He demonstrated that removal of the corpora lutea interrupted the normal events of pregnancy and that the blastocysts died in an otherwise intact animal.

At the same time, Bouin and Ancel (1910) observed an endometrial reaction characteristic of the effect of the corpus luteum, called "progestational proliferation." When the corpora lutea were removed in the rabbit, the endometrium showed no sign of this remarkable change.

In 1928 G. W. Corner began experiments that eventually culminated in the discovery of the progestational hormone. He ovariectomized female rabbits a few hours after ovulation and showed (1) that no progestational proliferation occurred in their endometria and (2) that the blastocysts, recovered 4 to 6 days later, had all died at the time they entered the uterus. Clearly, without corpus luteum function, progestational proliferation, implantation, and successful survival of the blastocysts could not occur. It is significant to remark here that the effect of estrogen and, subsequently, that of progesterone on the endometrium are the same in the rabbit, the monkey, and the human, showing no sign of species difference.

Allen and Corner (1929) obtained the first crude extract of the active principle of the corpus luteum, which looked, said Corner, "like a poor grade of

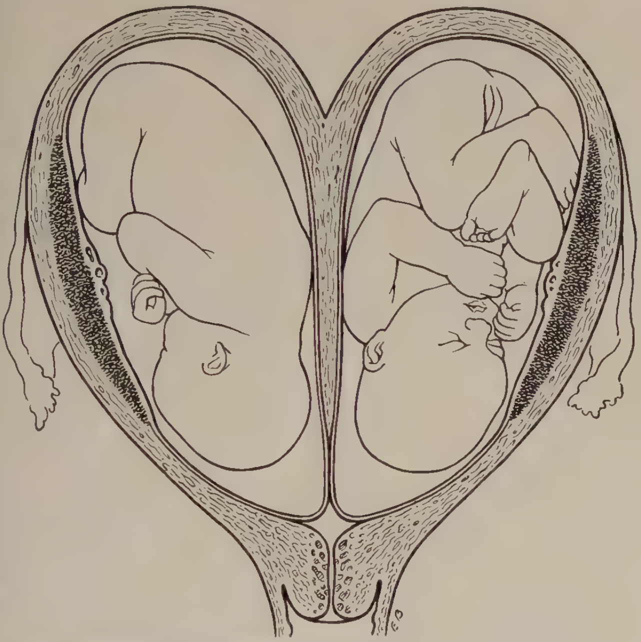

FIGURE 1. Twin pregnancy in a bicornate uterus. This coincidence is known to lead to ndependent deliveries from the same uterus, several weeks apart.

utomobile grease.” Whatever it resembled, however, it replaced all the known unctions of the corpora lutea. Corner and Allen showed that, in the rabbit, rogesterone not merely ensures the normal course of implantation, but also uards the conceptus throughout the entire duration of gestation. The emryos are resorbed, miscarried, or delivered prematurely if the corpora lutea re removed at any stage in gestation; but pregnancy continues undisturbed f the ovariectomized animal is treated with crude progesterone in sufficient uantity. Furthermore, Heckel and Allen (1939) demonstrated that pregancy in rabbits can be prolonged beyond its normal duration by progesterone reatment. Later, with synthetic progesterone, my associates and I were able o confirm Corner and Allen's classical experiments on the maintenance of regnancy (Csapo, 1955).

Knaus (1930) made the discovery that the smooth muscle of the uterus in he pregnant animal does not react to pitocin. He showed also that the corus luteum extract abolishes the pitocin effect in the nonpregnant animal. eynolds and Allen (1932) found that the contraction pattern characteristic f the estrous rabbit is eliminated by progesterone. We can thank Reynolds r formulating and describing in detail current problems concerning myoetrial function and endocrine regulation in his book *Physiology of the Uterus* 1949).

It should be emphasized that these now classic experiments were extensively nd successfully repeated. The quality of the descriptions, the clear presentation of facts, the absence of mere theoretical models and schematization a impressive. With these qualities, Gustav Born's strange legacy was develop to the point at which it could be turned over, in the form of progesterone crystals, to the obstetrician. These crystals promised to relieve human sufferin and guard normal pregnancies against uterine dysfunction.

The subsequent years brought unexpected disappointments. The 19 figures of Anderson, of the Johns Hopkins Hospital, Baltimore, Md., repo 10 per cent abortions and 7 per cent premature delivery. He estimates th one third of the abortions and two thirds of the premature deliveries were du to functional disorders of the myometrium, no anomaly being found (with t available laboratory methods) in the mother, placenta, or fetus to account f these incidents. The disorder had manifested itself by an error in timi only; clinically effective uterine contractility had merely begun several mont before term. Of course these functional myometrial disorders may not primary diseases of uterine muscle, but rather consequences of some error regulation that we are as yet unable to understand and correct. I sha present observations in support of the view that the synthesis or metabolis of progesterone by the placenta, a major endocrine gland during pregnanc may be involved in the endocrine imbalance, and that placental dysfunctio may be linked directly to this group of disorders. Clearly, the shameful ter "pregnancy wastage" applies annually to several hundred thousand inciden in the United States alone involving injuries and death related to pregnancy, a physiological process that becomes a disease too often only because we do n understand it. Significant as it may be in practical obstetrics to understan fully the endocrine control of the pregnant uterus, the theoretical significan of this problem is even greater. I am convinced that the ancient problem that of maintaining and terminating pregnancy, is the key to obstetrical physiology.

The striking discrepancy between laboratory success and clinical failure demands first an adequate explanation. Often I have felt that this startlin difference between the rabbit and the human, which will be the keynote of thi paper, has a significance much greater than its practical implication suggest The knowledge culminating in the discovery of progesterone justly was expected to become a powerful tool in controlling certain disorders of huma pregnancy. Had it worked, it would have been a striking example of the victory of research over disease. Experience, however, showed failure; worse it left both the obstetrician and the scientist with doubts and skepticism regarding the applicability of the theoretical to the practical. This is an unexpected defeat, which must be clarified for its own sake, and also because o its far-reaching consequences.

Did this discrepancy not lead to a new type of clinical research in obstetric that is being justified by the acceptance in full of the concept "species difference?" Did it not result in experiments in human subjects without sufficien preliminary studies in laboratory animals, which would have clarified th problem conceptually and technically? Are these experiments conclusive in spite of being greatly handicapped by such factors as the extreme complexity of human biology, the limitations of the moral code of medicine, the interference of disease, the modifying effects of the human psyche, and the diversity of patients? Can we expect to detect fundamental principles in such a complexity? Are we not creating a literature that is diffuse and uncertain, that teaches us to appreciate the superficial modifications of biological processes, and lets us overlook the fundamental principles? Do not the basic principles of reproductive function and regulation, which we attempt to understand eagerly and urgently, afford a foundation on which obstetrical physiology can be built? Is the convenient phrase, "species difference," invented to explain the discrepancy between the rabbit and the human, justified in the extent that it is used and abused? Is it at all likely that different rules govern in the rabbit and the human such a fundamental biological process as the maintenance and termination of pregnancy, or does this concept, as usually understood, provide a serious obstacle to the development of medical science? I believe in the second alternative, and I suggest that we very carefully consider this question, as well as the magnitude of our ignorance about the principles of myometrial function and regulation, in the light of some recent observations.

The question is not whether humans and rabbits are different. Of course they are; but are there significant differences between them in such fundamental matters as the conduction of a nerve impulse, muscle contraction, vision, circulation, metabolism, and energy production? With the help of molecular biology, as we become increasingly better equipped conceptually and technically to study the basic principles of function and regulation of living structure, we are given a chance to see, if we look for it, that fundamental principles are carefully preserved in the function of a variety of organs in different species. If so, why should we anticipate or actually claim species differences in the fundamentals of the maintenance and termination of pregnancy?

Once we use the word "fundamentals," however, we are prompted to ask what is known about the mechanism of the maintenance of pregnancy and about the role progesterone plays in this process. By asking this question I do not wish to add new phenomena to the excessive number of them we already possess, but rather to explain some of the old ones. This approach leads us to experiments in which classic endocrinology and physiology offer us a start only, and we must search for conceptual and technical help in biophysics and biochemistry. We must descend to the level of organization where biological functions actually proceed—the molecular level.

What little has already been done along these lines will be mentioned here only in brief to keep the mechanism of progesterone effect in focus. If we are nterested, however, in how progesterone acts, we must consider the structure, chemical composition, and characteristic function of the target organ on which this effect is exerted. Clearly, it is necessary to know a great deal more about the function whose regulation interests us than to speak simply of "contraction" and "relaxation," or of "uterine activity."

The smooth-muscle cell of the uterus is designed to develop force or to do work. To perform this task properly it must possess a contractile system. Our first question is: Of what is this final contractile system composed and how does it work? A long series of experiments (Csapo, 1948, 1949, 195 1955, 1958; Blasius and Schuck, 1955; Kominz and Saad, 1956; Cretius, 195 revealed that the contractile system of the myometrium, like that of cros striated muscle, is composed of the proteins actin (A) and myosin (M), aden sine triphosphate (ATP), and ions (FIGURES 2 and 3). We have isolated the compounds from the uterus and have found that, when put together *in vitr* free of the complexity of the myometrial cell, this highly simplified syste contracts like muscle. This observation reveals not only the constituents the final contractile system of the myometrium, but also the significant fa that the phrase "species difference" loses its meaning at this level of organiz tion, since no significant differences could be detected in the contractile system isolated from human, rabbit, rat, and cow uteri on the one hand and from variety of cross-striated muscles on the other. This in turn implies that th mechanism of contractility also is similar in a variety of muscles.

Our next question is: Where is this contractile system in the intact musc cell? The myometrium is composed of fiber bundles about 100 μ in diamet that are made up of spindle-shaped cells about 10 μ diameter (FIGURE 4). Bo ler (1938) showed that these bundles contract in an "all or none" fashion, lik syncytia, and develop polarity in a DC field, implying that they might be su rounded by an excitable membrane of their own. The individual cells withi the bundle, each surrounded by an excitable membrane, are considered at pre ent as the final physiological units of the myometrium. The nucleus is foun in the middle of the cell and the myofibrils occupy most of the plasma spac Between the myofibrils we see, with the electron microscope, the mitochondri spaced like beads in a row. Thus the site of energy production is in immediat contact with the site of expenditure. Our main interest here, of course, is th myofibril composed, as revealed by electronmicroscopy (Csapo, 1955), of bundle of individual filaments made up of the contractile proteins themselve This is shown by the fact that ghost muscles from which actomyosin (AM) i extracted have no filaments within them (Huxley, 1956). The ultrastructur of these filaments is not yet known, nor are the exact chemical and physica changes that take place when these filaments pass over from the resting t the "active state," followed by the mechanical response. It is possible, how ever, to detect the active state during mechanical latency, as well as to recor the mechanical events quantitatively (Csapo, 1955).

One of the most startling observations in my experience with muscle (Csap and Goodall, 1954; Csapo, 1955, 1956a) was the finding that the rabbit uterus like the classic muscle, the frog sartorius, obeys accurately the "length-tension" "strength-duration," "threshold," and "load-shortening velocity" relation ships. These relationships are the classic pillars of muscle physiology, well summarized by the widely known equation of A. V. Hill, the British biophysi cist. It is startling, indeed, to learn that the uterus obeys Hill's equation considering that the formula was derived from experiments with the sartoriu muscle, which makes it possible for the frog to jump quickly enough to catch the fly.

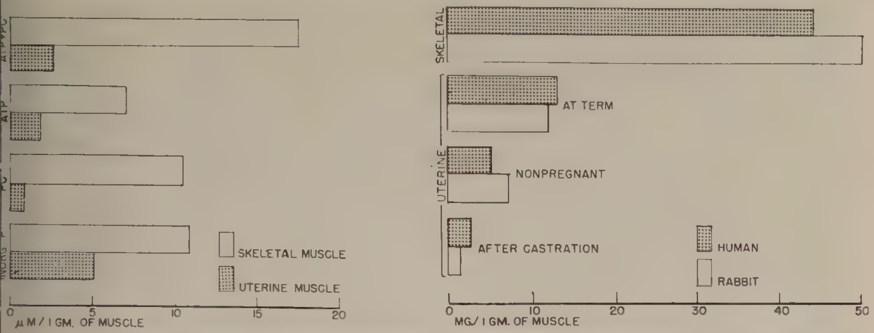

FIGURE 2. The actomyosin and high energy phosphate concentrations of cross-striated nd uterine muscle.

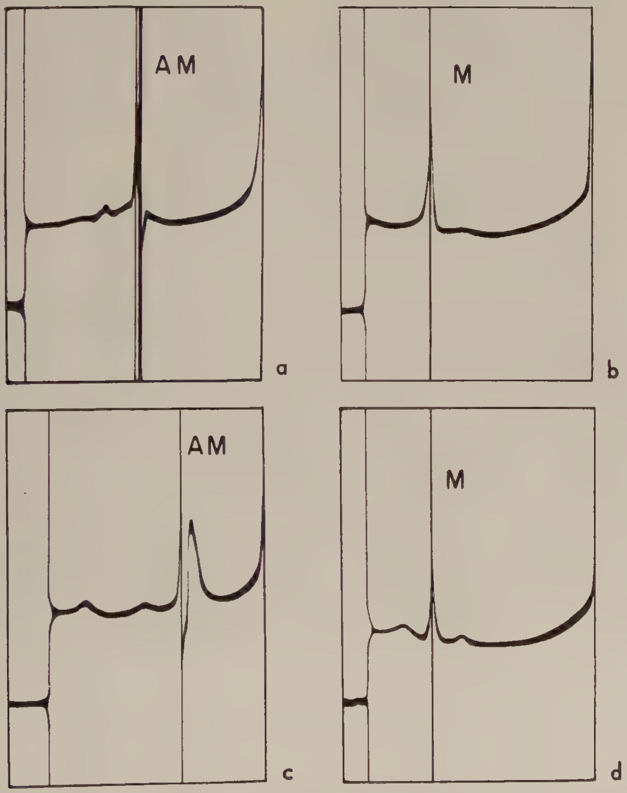

FIGURE 3. The sedimentation of actomyosin (AM) and myosin (M) in the ultracentrifuge (Kominz and Saad, 1956). (*a*) Rabbit skeletal muscle, (*b*) with ATP; (*c*) human uterus at term, (*d*) with ATP. KCl = 0.5 M, $MgCl_2 = 10^{-3}$ M, ATP = 5×10^{-3} M. Time of exposure 64 to 114 min. after reaching 59,780 rpm. Protein concentration = 0.7–0.9 per cent. Sedimentation rates: (*a*) = 8.5, (*b*) = 3.9, (*c*) = 9.3, (*d*) = 4.4. Note the characteristic hypersharp M peaks in the presence of ATP.

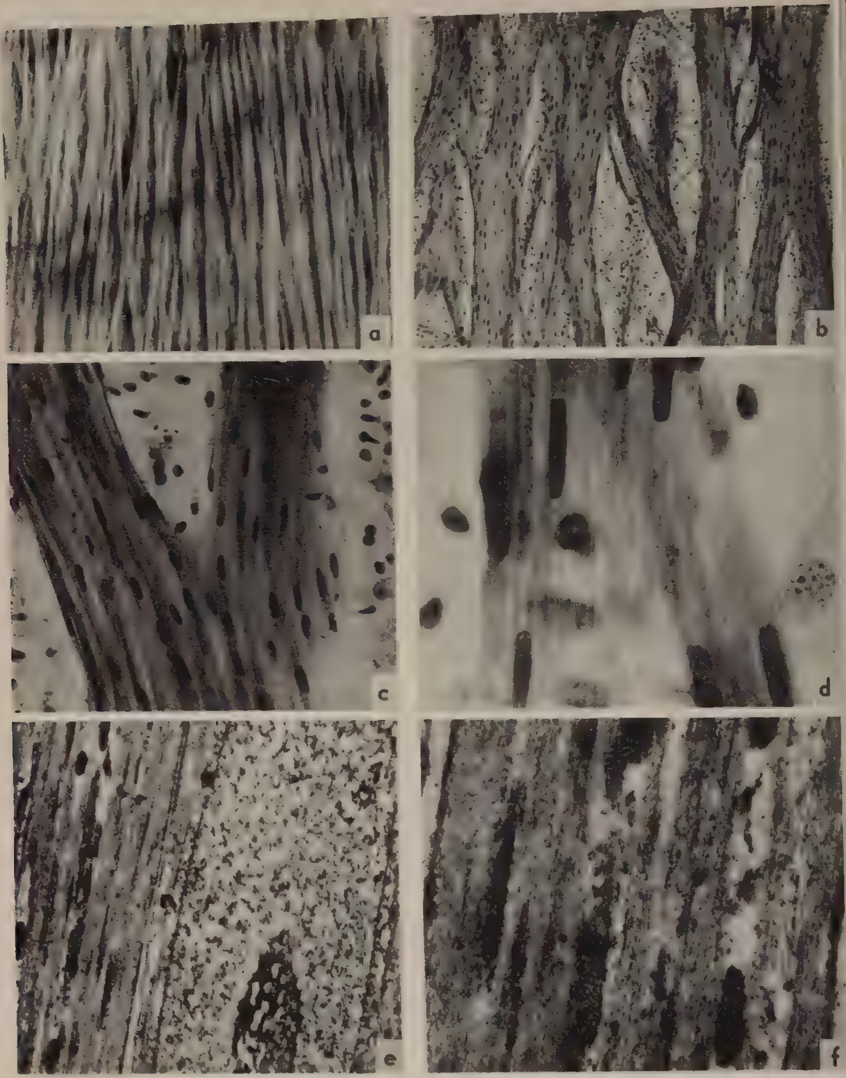

FIGURE 4. Structure of the longitudinal muscle layer of the pregnant rabbit uterus at term. (*a* to *d*) Light microscopy, ×12, ×75, ×300, and ×1,000, respectively; (*e* and *f*) Electronmicrographs, ×10,000 and ×20,000, respectively.

Work or tension of the myometrium is a function of its AM concentration (Csapo and Corner, 1953). The [AM], on the other hand, is determined by estrogen (Csapo 1948, 1949, 1950, 1955; Blasius and Schuck, 1955; Cretius, 1957). The uterus of the immature rabbit, or that of the ovariectomized rabbit or human, has a very low [AM], which can be increased by estrogen treatment to the value characteristic in natural estrus (FIGURE 5). A further ncrease takes place during normal pregnancy, which is reversed in the puerperium. Parallel with changes in [AM], the maximum tension of the intact uterus changes also, keeping the relationship of tension to [AM] constant. From viscosimetric observations, it appears that the extraordinary task of delivery is accomplished by an increase in the [A] of the uterus, which in turn results in more AM and a consequent increase of the maximum working capacity (FIGURES 6 and 7).

Whether the myometrium, in fact, performs maximum function depends on a number of additional factors, such as sufficient concentration of high-energy

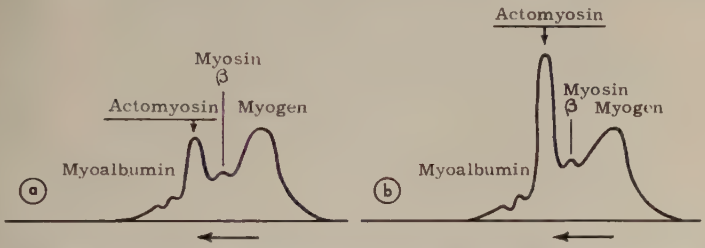

FIGURE 5. Electrophoretic patterns of uterine muscle proteins (after Blasius and Schuck, 1955). (*a*) Uterus of immature rabbit; (*b*) the same after estrogen treatment. Note the increase of the AM peak after estrogen treatment. Courtesy of *Klinische Wochenschrift*.

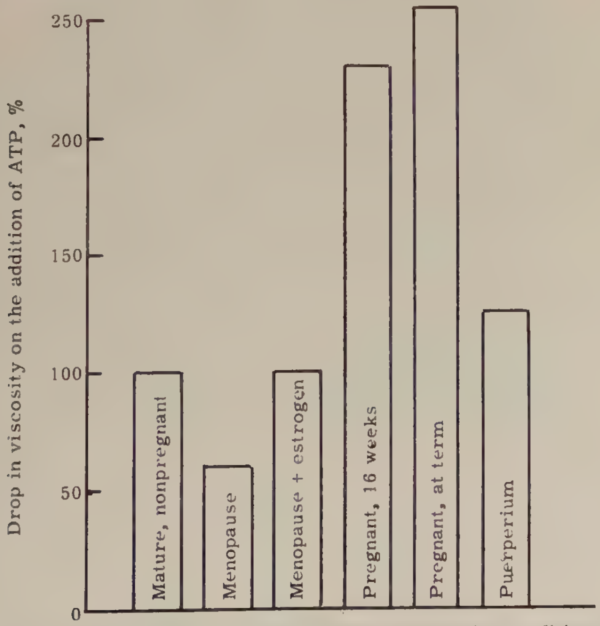

FIGURE 6. Actomyosin of the human uterus in different endocrine conditions (after Cretius, 1957). Note the decrease in AM after withdrawal of estrogen and its increase on estrogen administration; note also the further increase in AM during pregnancy and its decrease after parturition. Courtesy of *Gynaecologia*.

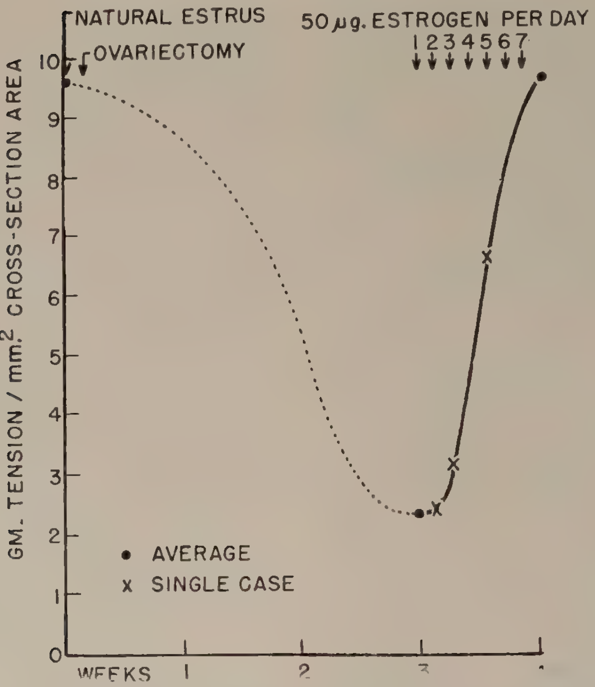

FIGURE 7. Effect of estrogen on the maximum isometric tension of rabbit uterine strip *in vitro*. Note the decrease in tension following ovariectomy and the return to the origina (estrus) value after estrogen treatment. Tension in natural estrus = 9.6 gm. ± 1.11 SE/mm cross section area; the [AM] = 7.6 mg./gm. of tissue. Tension after ovariectomy = 2.3 gm. ± 0.41 SE; the [AM] = 1.7 mg. Thus, tension/[AM] remains constant in the two different endocrine states; 1.27, 1.35, respectively. Courtesy of *Science*.

phosphate (∼ph), that is, ATP + CP, proper ionic gradients across the excitable membrane of the cell, or effective "excitation" and "excitation-contraction coupling" (FIGURES 8 and 9). If the CP of the uterus is exhausted or the ionic gradients across the cell membrane are eliminated, the myometrium becomes "inexcitable" electrically or pharmacologically because the normal membrane mechanism for excitation is eliminated. Such a uterus remains contractile, however, as long as the ATP store is not altered significantly and responds readily to a strong electric field or quick cooling. These stimuli are believed to activate the contractile system directly (Csapo, 1958).

These points are mentioned to call attention to the fact that the uterus, as well as other muscles, may be held in complete or relative inactivity even though it possesses maximum contractile capacity. Under progesterone domination, the uterus is such a muscle; in many respects it is similar to a procaine-blocked nerve or cross-striated muscle (Csapo 1956a and 1956b). What, then, is known about the effect of progesterone on the myometrium?

First, progesterone does not affect the final contractile system. Whether the animal is or is not treated with progesterone, the [AM] and the contractile

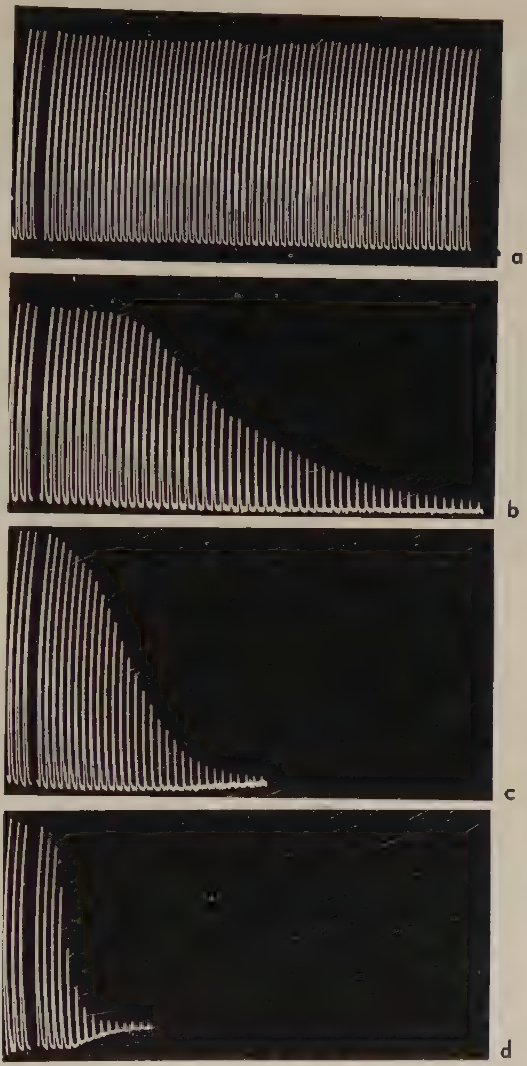

FIGURE 8. Maximum tension as a function of the $\sim$ph content of the rabbit uterus. Four identical segments from the same rabbit uterus, in natural estrus, are tetanized once every minute. (*a*) Krebs solution, 95 per cent O_2 + 5 per cent CO_2; (*b*) M/10,000 IAA is applied; (*c*) O_2 off, N_2 on; (*d*) IAA + N_2. Note that tension declines more rapidly as the resynthesis of $\sim$ph is increasingly blocked. Courtesy of Butterworth Publishers, London, England.

capacity are at the values determined by the estrogen effect. If the uterus is dominated by progesterone, however, the normal electrical or pharmacological stimulus is relatively ineffective, and maximum tension can be obtained only by stimulating the membrane simultaneously at all points or by directly stimu-

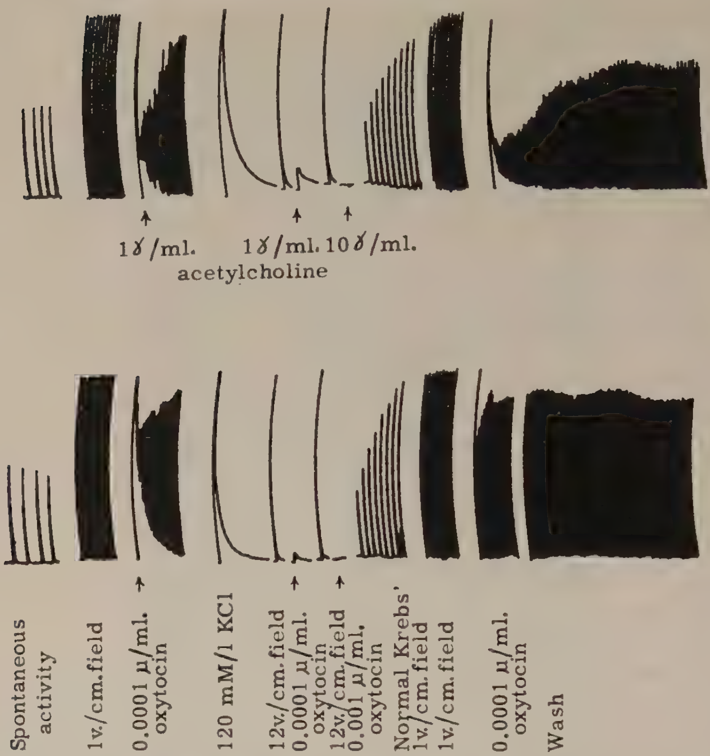

FIGURE 9. The maximum "myoplasmic excitability" and the lack of pharmacologica reactivity of the K-depolarized rabbit uterus at term. In a modified (120 mM/1 K and 35 mM/1 Na) Krebs solution, the uterus develops a transient contracture followed by rest. In the absence of K and Na gradients and membrane potential, maximum tension develops repeatedly in a longitudinal AC field of sufficient strength (12 v./cm.). Note the lack of pharmacological reactivity in spite of maximum contractile capacity. Note also complete recovery on repolarization and return of normal excitability (1 v./cm.) and pharmacological reactivity.

lating the contractile machinery (FIGURE 10). Such stimuli, of course, do not exist under physiological conditions, but their theoretical value is considerable. If the estrogen-dominated uterus is stimulated effectively at any point along its length through the physiological membrane mechanism, the organ will respond as a whole because the myometrium in this condition propagates the excitation wave in all directions. It is this propagation that fails under progesterone domination, so that the stimulus remains localized. The myometrium is "blocked" by progesterone and will not conduct the excitation wave. Thus, activation and the subsequent mechanical response remain partial, and the organ as a whole is inefficient in delivering its contents. The progesterone-dominated uterus is not quiescent, however. It is active, and spontaneous activity is generally more frequent than in the uterus under estrogen domination. The uterus even may be in slight sustained contrac-

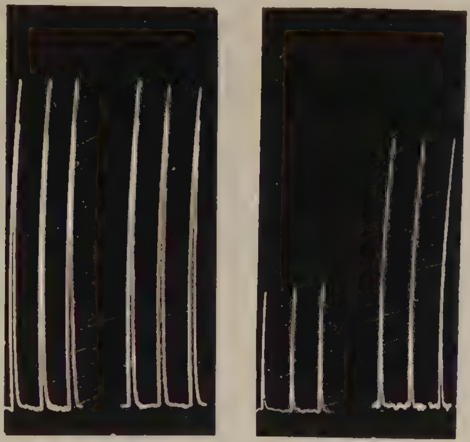

FIGURE 10. Progesterone "block." From left to right: isometric tension, developed by the estrogen-dominated rabbit uterus when the strip is stimulated simultaneously at all points along its length; only one half of the muscle is stimulated; uterus is dominated by progesterone and only one half of the strip is stimulated; the same strip stimulated at all points simultaneously. Note that under progesterone domination only the stimulated portion of the muscle is activated, due to the lack of propagation. Courtesy of Butterworth Publishers, London.

ture. It is the over-all and synchronized activity which fails to develop, and all experiments seem to indicate that, as long as progesterone effectively blocks the myometrium, this condition is maintained and cannot be suspended except by elimination of the effective progesterone block.

The mechanism of the progesterone block is not yet clearly understood. The condition can be simulated by treating the estrogen-dominated muscle *in vitro* with a modified Krebs solution containing excess K and low Na. The analogy suggests that, under progesterone domination, both the K and Na gradients across the excitable membrane are reduced. This alteration will raise the threshold and increase the rate of spontaneous activity and contracture, and also will reduce or eliminate pharmacological reactivity and conduction, producing a state characteristic of the progesterone-dominated myometrium (FIGURE 11). The analogy is further supported by the observation of Hovarth (1954) that, in the progesterone-dominated uterus, the $[K]_i$ decreases, whereas the $[Na]_i$ increases, resulting in a decrease in both ionic gradients. Although the shift is in the right direction, the extent of the change is not sufficient to account fully for the observed physiological behavior. At least one other ion must be considered in the effect, and we centered our interest recently on the Ca ion because it has striking effects on both excitability and mechanical response and seems to be involved directly in endocrine regulation.

The available data do not allow us to distinguish between two possible mechanisms of the progesterone block. It might be due to a reduction in membrane potential below a certain "critical" value, where excitable tissues

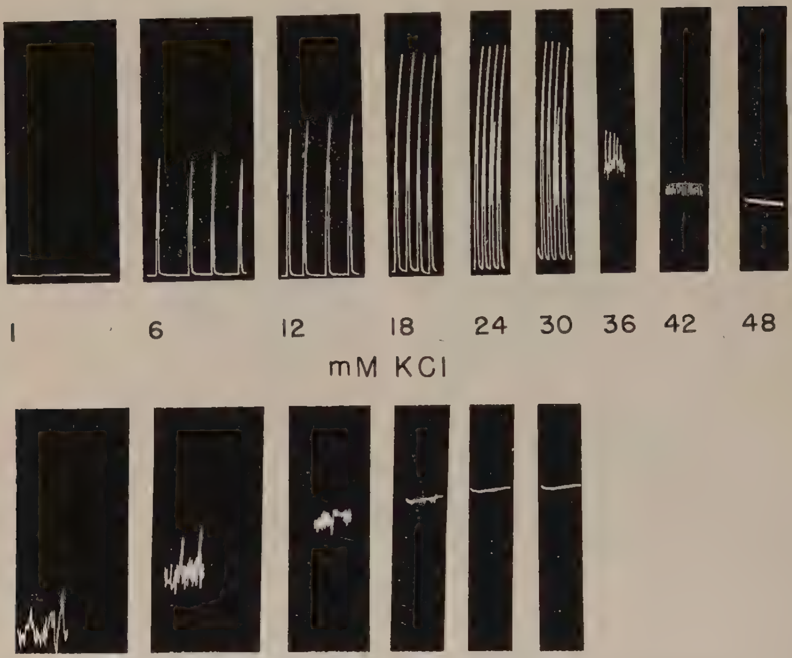

FIGURE 11. Effect of depolarization and hyperpolarization on the "spontaneous activity" of the estrogen- (upper row) and the progesterone- (lower row) dominated rabbit myometrium. Note that depolarization increases the spontaneous activity of the estrogen-dominated uterus in the range of $[K]_0 = 6$–30 mM. Above 30 mM the estrogen-dominated uterus behaves as the progesterone-dominated uterus does at the physiological $[K]_0 = 6$ mM. Hyperpolarization ($[K]_0 = 1$ mM) abolishes spontaneous activity. Courtesy of Academic Press.

in general cease to propagate. It is also possible, however, to visualize a block without significant change in membrane potential, like the procaine block on nerve and muscle. Since the advance of delivery is known to be accompanied by an increase in spontaneous activity and pharmacological reactivity, propagation must be restored and the threshold for effective excitation lowered. This can be accomplished by a slight increase in membrane potential if it was previously lowered below the "critical" value, or else by a slight decrease in membrane potential if it was originally high. The microelectrode technique can distinguish between these two alternatives.

Without further detail, we may summarize as follows our present understanding of myometrial function and its endocrine regulation. The uterus, insofar as it has been investigated, is known to possess all the characteristic qualities of excitable and contractile tissue. As yet there is no reason to regard this organ as different in molecular composition or in the principles of its function from other members of the large family of contractile tissues. There are quantitative differences and differences in kinetics, however, from one muscle to another, which allow the muscle family to cover a colorful variety of physiological functions. This variety in function, however, disappears when we compare uteri of different species, provided our methods are adequate and standardized. It is dangerous to compare results obtained in different laboratories under different or ill-defined experimental conditions because seemingly insignificant variations in technique will yield completely different results, for which some would account by species difference. If we cannot find significant variations in structure, chemical composition, and mechanical and excitable properties in the uteri of different species under well-defined conditions, we can justly doubt the existence of differences in the principles of regulation. It was this belief that stimulated us in attempting to clarify the general role of progesterone in the maintenance of pregnancy. We do not know of any substance other than progesterone that, in a physiological concentration, effectively blocks uterine function. Since progesterone in some way is clearly involved in the maintenance of pregnancy, I propose here to list evidence in support of the concept that it is the progesterone block which accounts best for the maintenance of pregnancy in different species, and that the withdrawal of progesterone block is responsible for the onset of labor.

Concerning the maintenance of their pregnancy, laboratory animals such as the rabbit and the human subject are believed to belong to two characteristically different groups (Courrier, 1945). In humans the ovaries are not needed for the maintenance of pregnancy from the third month of gestation onward, whereas in rabbits the ovaries are indispensable through the duration of gestation. Because the corpora lutea of the ovaries are known to be the source of progesterone, this difference in ovarian function in different species was taken as evidence that the maintenance of pregnancy is secured differently from one animal to the next. When progesterone was isolated from the human placenta (Salhanick *et al.*, 1952), however, only a difference in source could be claimed, not a difference in the mechanism of the maintenance of pregnancy. In certain animals termination of gestation was controlled in the ovaries and in others by the placenta, but the mechanism of control—progesterone—was the same in both.

Experiments in our laboratory (Csapo, 1955), however, showed that the later the rabbit is ovariectomized, the longer its pregnancy is maintained, suggesting that at the end of gestation, even in the rabbit, the placenta partially takes over ovarian function. This observation also implies that, as a rule, laboratory animals shift from ovarian to placental dominance to a greater or lesser degree in the endocrine control of gestation, and that the placenta has a key role in the termination of pregnancy. Recent studies in our laboratory confirm this. In these experiments we reversed the approach; we destroyed placental function in the rabbit by introducing 1 ml. saturated NaCl solution into the anmiotic cavity in order to dehydrate the placenta. This treatment resulted in premature delivery, although the ovaries remained intact. Thus, the ovaries alone are insufficient to maintain pregnancy in the rabbit at a late stage of gestation. The assumption that insufficient progesterone production was responsible for the effect was supported by the fact that, in these animals with destroyed placental function, progesterone substitution therapy was effective in maintaining or even prolonging gestation for any desired length of time.

What, then, is the evidence against the conclusion that progesterone occupies a key role in the maintenance of human pregnancy in the same general fashion as is known to be the case in the rabbit? The most readily accepted argument is the disappointing failure of progesterone treatment in human subjects. To me, however, this is the least convincing evidence, since I suspect that, more often than not, we treat the wrong patient, or use inadequate doses and at inappropriate times. Statistical analysis of even a large group of patients cannot clarify this point. The treatment can be expected to be effective only if the disorder is restricted to insufficient progesterone production or excessive metabolism. We do not know how to select these patients. Calculations suggest that the effective dose range is 100 to 200 mg. progesterone per day, and the latency period 12 to 24 hours. Thus we perhaps give too little and too late, even for those patients who might be cured by progesterone.

The long latency of progesterone effect suggests that progesterone might not act on the myometrium as we know it and look for it in our chemical and biological assays, but in some modified form yet unknown. This possibility and the lack of knowledge about equilibrium conditions between the endocrine organ, the blood, the target tissue, and the urine call for caution in the functional interpretation of tissue, blood, and urine analysis of progesterone and its metabolites. It is significant to remember that Zarrow and Neher (1955) found no progesterone in the blood of the rabbit 5 minutes after I.V. injection of the steroid, yet pregnancy is known to be maintained indefinitely by progesterone therapy. This can mean only that we look for progesterone either in the wrong place or in the wrong form. Frankly, I should not know how to interpret a rise or fall of blood progesterone in terms of myometrial function, because it does not tell whether the myometrium is giving or taking up progesterone.

Let us assume for the moment that the progesterone production of the human placenta and the effect of this steroid (or that of its modified form) on the myometrium are responsible for the maintenance of pregnancy. Let us assume further that the cessation of an effective progesterone block results in the termination of gestation. If that is true, how is it possible for human twins to be born 2 months apart from the same bicornate uterus?

I have proposed a theory (Csapo, 1956b) that involves the hypothesis that the placenta gives up its progesterone, not to the blood stream, as all endocrine glands are believed to do, but by direct diffusion to the neighboring myometrium. The result of this would be a progesterone concentration gradient in the myometrium with a peak at the placental implantation site, and therefore a localized effect (FIGURE 12). This theory was tested on the rabbit, using a series of biological tests involving mechanical and excitability studies of the excised myometrium. The expected difference in progesterone domination between placental and interplacental sites in the rabbit close to term should be less obvious than in the human, because of effective ovarian

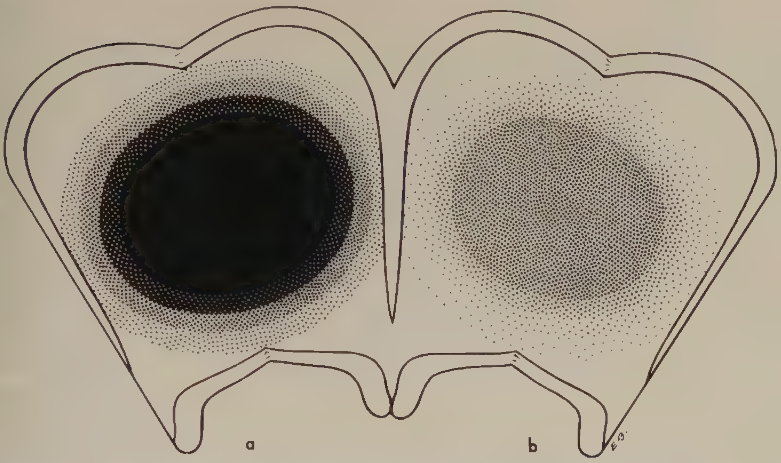

FIGURE 12. Local effect of placental progesterone on the myometrium (hypothetical illustration). (*a*) Placental progesterone synthesis continues; (*b*) synthesis ceases.

function. Nevertheless, it was observed that the myometrium was more strongly dominated at placental sites by progesterone than it was between these sites. Clearly, the placenta has a local effect on the myometrium, and this localization may be greater in species in which the corpora lutea regress at an early stage in gestation.

It is reasonable, then, to regard this local effect of placental progesterone as decisive in the maintenance and termination of gestation, through the maintenance and cessation, respectively, of the progesterone block. If one placenta in a bicornate uterus fails to produce an effective block, the uterine horn around it must be activated as a whole and must deliver its contents while, in the other horn, having an effective block sustained by the other placenta, pregnancy must continue. This explanation does not, of course, rule out the possibility that substances other than progesterone are involved in determining the activity of the myometrium. It is strongly implied, however, that a decisive role is given to a *local* factor that appears to be the progesterone block.

Whether this theory is completely correct cannot be claimed as yet. However, it serves its purpose well in indicating new and suggestive experiments. First, J. C. M. Browne of London, England, with whom I had the pleasure of planning an experiment, advised me that he has succeeded in maintaining human pregnancy beyond term by massive progesterone treatment. Second, my former colleague L. Ph. Bengtsson of Lund, Sweden, informs me that abortion induced by formalin injection into the amniotic cavity can be postponed by massive progesterone treatment. He found also that pregnanediol in the urine declines rapidly after the formalin injection, whereas its concentration remains high if progesterone treatment is employed at the same time. When progesterone treatment stops, pregnanediol falls; abortion generally begins when the 24-hour pregnanediol drops below 10 mg. There seems to be a good correlation between oxytocin response, cessation of progesterone treatment, and drop in 24-hour pregnanediol.

I was once asked to explain, on the "progesterone block" principle, the occasional success in inducing premature delivery with Pitocin treatment. I believe that success here is not due to the characteristic effect of Pitocin on the myometrium, for the effect of this compound is immediate, whereas induction of delivery generally takes several hours, if not days, except at term. Bengtsson (1957) did an experiment in our laboratory that is relevant to this problem. He injected rabbits about the twenty-second day of gestation with Pitocin and, after different time intervals, studied the uterine contents. He found hemorrhages, varying from microscopic to massive and completely destructive, *within* the placenta and not between the uterus and the placenta. Such bleeding might interfere with endocrine function and with the progesterone block. If the block is removed, the myometrium becomes active. Unfortunately, when this treatment is employed, one must consider the possible harmful effects of placental hemorrhages on the fetus, a serious practical consequence. Our knowledge concerning the mechanism of oxytocin effect on the myometrium has improved considerably in recent years, but space does not permit me to discuss this interesting subject here.

Parenthetically, we should mention the possibility, not yet explored experimentally, that estrogen also participates in the maintenance of pregnancy by increasing the "excess" or "safety" potential of the excitable membrane of uterine muscle. This effect would reduce or eliminate spontaneous activity, the driving force of the myometrium. This effect also can be a localized one.

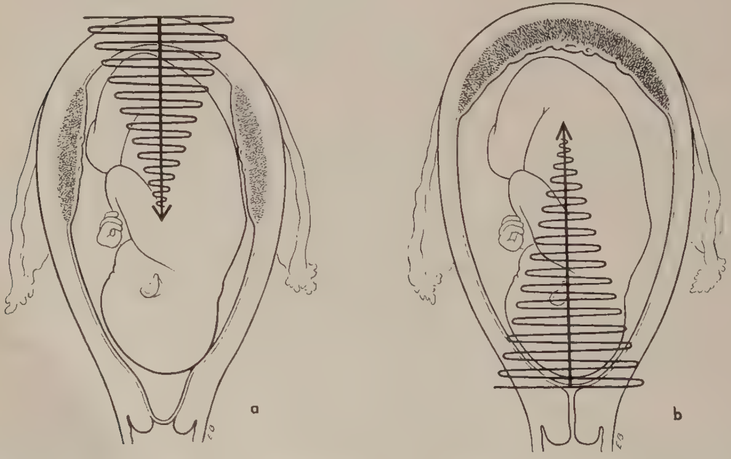

FIGURE 13. Consequences of a progesterone concentration gradient in the parturient myometrium determined by the location of the placenta (hypothetical illustration). (*a*) The corpus recovers first from the block, resulting in a downward activity gradient; (*b*) the opposite situation.

However, even if estrogen exerts such an effect, the progesterone block should be kept the center of interest. H. Jung of Freiburg has informed me (personal communication) that action potentials disappear at the placental implantation sites, a finding that can be explained best by the local progesterone block.

The significance of the local effect of placental progesterone does not vanish when labor begins. The location of the placenta in the uterine cavity should be considered seriously as a possible source of uterine dysfunction in labor. Along these lines I can as yet only offer suggestions.

If the placenta is located at or below the midline of the uterus we should expect the fundus to recover first from the progesterone block when progesterone synthesis ceases. Thus, activity should begin at the fundus, providing a normal "activity gradient," described by Reynolds (1949), that is, a downward gradient toward the cervix (FIGURE 13). If the placenta is located high, the lower uterine segment will first recover from the block, and the activity gradient will point in the wrong (upward) direction. In the early phase of labor, this reversal of the activity gradient will manifest itself clinically only when it becomes clear that in spite of satisfactory labor pains there is no progress. If the theoretical assumptions here are essentially correct, it follows that any attempt to force the uterus in this condition to deliver its contents is wrong for the following reasons. If the attempt is successful and the fetus is delivered by a partially blocked myometrium, during or after the delivery of the placenta serious bleeding may occur as a result of the partial block. If, on the other hand, the fetus cannot be delivered successfully, the myometrium may be exhausted by forced labor when the block is withdrawn in the fundus, and the activity gradient may turn automatically from the wrong to the right direction.

Until we can clarify the correlation between placental location, progesterone block, activity gradients, and uterine function or dysfunction in pregnancy or during and after labor, conservative obstetrics appears to be the safest course to follow. The well-known proverb of conservative obstetrics, to "wait and wait patiently," seems well justified on theoretical grounds.

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ELECTROHYSTEROGRAPHY

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In the human, uterine contraction during labor is associated with electric activity that can be recorded from various points on the body. Records have been obtained from electrodes applied to the abdomen alone, to the back, to the extremities, to the cervix, and to the uterus exposed at cesarean section.

This activity consists of two types of waves. One consists of irregular diphasic waves in a frequency range of ½ to 2 cps and amplitudes up to 500 μv. The second form consists of very slow waves, generally but not always diphasic, of a duration of ½ to 2 min., and amplitudes of 2 to 25 mv. Since it is difficult to record both of these forms of activity on the same recorder, two different types of apparatus have been used. One of these is designed to record primarily the slow activity—a push-pull direct-coupled amplifier driving a pen writer. This equipment also records the faster activity, but the characteristics of the system are such that these waves are seen very poorly. The second type of apparatus is a push-pull amplifier driving a magnetic pen writer, with characteristics so designed that the slow waves are not recorded, while the faster waves are well seen. In effect, then, it is possible to study the two types of electric activity separately.

The slow waves.[1-7] Each contraction is associated with a single, large slow wave, which is superimposed on the ever-present "standing potential." The wave begins shortly after the contraction becomes apparent externally, although it may well begin simultaneously with internal uterine contraction. There is generally a deflection of 2 to 10 mv. in one phase, followed by a deflection in the opposite phase of equal or greater amplitude. The duration of the first phase is about 15 sec., the second phase, 1 to 2 min. The total duration of the wave may be equal to the total duration of contraction, or longer. The standing potential after each wave usually is different from that existing before the wave.

The shape of the wave varies considerably from one contraction to another. When recordings are made simultaneously from several points on the abdominal wall, it is found that, in early labor, electric activity may exist at only one point, but is present at all leads when strong labor is established. The waves will differ in shape from one contraction to the next, but for any one contraction the wave shape will be the same in all leads.

This type of activity appears to be related to uterine activity in this way: the initial deflection is a gross resultant of uterine muscle contraction; the second deflection is a resultant of recovery from this activity. Some of the second deflection may come from the chemical activity of the uterus, and some of it comes from a general body response to (1) the muscle action and (2) the altered electric state of the body.

The faster waves.[8-11] Each contraction of normal labor is associated with a series of waves at a frequency of $\frac{1}{5}$ to 2 cps and an amplitude of 100 to 500 or more μv. This activity is entirely reproducible from one contraction to the next. It begins several seconds before the contraction can be recorded externally. The duration is commensurate with the duration of clinical contraction, being short in early labor and long in the later stages. It bears a constant relation to the contraction itself and to the pain of the contraction. Electric activity of this type ends as the relaxation phase begins. Pain is felt about 10 sec. after the electric activity begins, and ends simultaneously with the electric activity.

When recordings are made simultaneously from 3 areas on the abdominal wall (near each cornu and in the midline of the fundus), only 1 area will show electric activity in early labor, 2 areas will show activity later, and all 3 will show it in good, active labor. It is possible in some instances to record what appears to be transmission of a stimulus, in that 1 area shows electric activity, then the second, and then the third, the time between onset at the first area and onset at another being 5 to 10 sec. In normal active labor all 3 areas show activity simultaneously.

These findings serve two functions. They may be used to speculate, from the physiological point of view, upon the kind of muscular action that is taking place. They may also be used to study labor in a clinical sense, in order to differentiate various types of labor.

Physiological implications. The slow waves reflect a rather large-scale alteration of the surface standing potential. They are related in time to contraction and recovery. It seems reasonable to believe that the first phase is produced by contraction, that it is the sum of all of the electric potentials produced by the uterine muscle. The second phase, while related temporally to recovery, is probably complex. Some of it may be due to local chemical changes, but some is certainly due to a general shift in the body's electric field secondary to the initial phase.

The faster waves are clearly related to the contraction phase alone, and are not seen during relaxation or in the interval between contractions. They increase in amplitude as the contractions become stronger mechanically. They represent the basic electric activity from which the initial phase of the slow wave is derived. Each wave probably represents the summation of electric activity of a group of fibers.

Clinical implications. Early labor often is associated with electric activity at one or two of three areas, whether this is recorded as the fast or the slow activity. Normal active labor, progressing well from a clinical standpoint, is associated with activity at all three leads. This activity can be correlated with the strength of the contraction, in that the greater the force, the higher the electric amplitude. Thus, both early and active labor are associated with electric activity, which varies quantitatively but not qualitatively.

Uterine inertia has been studied only by means of apparatus that can record the fast activity. I chose this method because these waves are reproducible from one contraction to another, while the slow waves are not.[5, 8]

Those who have used the slow-wave apparatus[3, 4, 6, 7] have not studied abnormal labor sufficiently to make any definitive statements. Uterine inertia, a clinical state, exists when labor has begun, but has not progressed according to the normal pattern. These cases show two different types of record. About 90 per cent show electric waves of a "normal" form, at a frequency of ⅕ to 2 cps, but with low amplitude and short duration. These cases can be regarded as in normal but weak labor. Eventually, after a rather long time, they will deliver; also, they will respond gratifyingly to Pitocin, and deliver in a much shorter time. In the remaining 10 per cent, although the uterus becomes hard and the patient complains of much pain, no electric activity is recorded from the electrodes on the abdominal wall. These patients never dilate, and they do not respond to Pitocin. Clinically, then, two different states are found to exist, each with its own type of record. By means of the electric record it is possible to determine which cases should be treated by stimulation and which will require cesarean section.

The physiological and the clinical implications can be fused as follows: a normal uterine contraction consists of contraction of groups of muscle fibers of a considerable size, some groups beginning to contract simultaneously in all parts of the uterus, the remaining groups following within a short period of time. Contraction ends simultaneously throughout and a period of relaxation begins.

Early labor consists of contractions occurring in only one area of the uterus. As labor progresses, the impulse to contract is transmitted from the original area to other portions of the uterus. When strong labor is established, all areas of the uterus contract simultaneously, and now no pacemaker activity can be demonstrated.

In weak labor, contraction occurs in relatively small groups of fibers for only a short period of time. Whether every group of fibers in the uterus contracts weakly every time, or whether some contract on one occasion and not on another, cannot be determined by this method. The response of these patients to stimulation, however, suggests that all of the fibers are capable of strong contraction.

Finally, in those patients who have no electric activity and do not respond to stimulation, the size of the contracting groups of fibers apparently is so small that the resultant electric potential fails to reach the surface of the abdomen. Clinically, this is a serious disorder of uterine action. Physiologically, it must also be a serious disorder.

Turning finally to the uterine fiber itself, in normal labor the contractile system, the energy-supplying system, and the membrane potential are all in the ideal state for contraction. In early labor only some fibers are prepared for contraction—the contractile system and the energy system are prepared in all fibers, but the membrane potential is not yet at a proper value in all. Thus only some fibers contract. As time passes, the potential changes associated with their contraction are transmitted to the remaining fibers, some of which are stimulated to contract. In time, the condition of normal active labor comes to exist.

Observations on false labor show that this situation is not irreversible. Activity, once begun, can stop, the uterine fiber returning to the state in which it exists during pregnancy.

In weak labor, one of two situations exists. The general contraction is weak because only a relatively small number of fibers contract at a given time. This means that preparation for contraction is deficient, or that the membrane potential is held at too high a level for contraction to take place. These may both mean the same thing in some cases, in that insufficient food for the energy-supplying system conceivably can produce both conditions. The fact that an adequate supply of glucose and water corrects many of these cases suggests that it is the energy-supplying system that is at fault. Response to stimulation with Pitocin, however, suggests that simple alteration of the membrane potential may be all that is necessary; that the fibers have not yet reached the potential that allows rapid and rhythmic contractions. It may be argued that patients presenting these conditions are deficient in oxytocin. The electric studies adduce no data on this particular question, but a characteristic of these cases is early uterine exhaustion when Pitocin is added. If uterine atony and hemorrhage postpartum are to be avoided, Pitocin must be given carefully and must be continued in the third stage. Preferably, use of Pitocin should be preceded by use of glucose and water. The conclusion that the fiber is deficient in its energy-supplying system is inescapable.

In the severe form of uterine inertia, which does not respond to rest, glucose, water, or stimulation, the fiber is not prepared for adequate contraction. Small groups of fibers contract, but in such a disordered pattern that electric waves of a very small size are produced—too small to reach the abdominal surface. The remainder of the fibers remain quiet, as during pregnancy. Such patients are not in physiological labor, but the contractions continue and treatment must be provided clinically. This necessity for treatment has prevented adequate experimental observation of these patients. If sufficient time could elapse, all of the fibers probably would reach the chemical state necessary for adequate contraction. Continuation of such an abnormal labor results in stillbirths and maternal infection and exhaustion; therefore the time necessary for this presumed change can not be determined.

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OXYTOCIN AND CONTRACTILITY OF THE PREGNANT HUMAN UTERUS*

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Methods for Recording Uterine Contractility

Quantitative Study

The contractility of the pregnant human uterus is measured through the recording of the amniotic fluid pressure[1, 2] by means of a thin polyethylene catheter introduced into the amniotic sac through the anterior abdominal wall and connected with a Sanborn electromanometer, model 121C, which is plugged to a Sanborn Poly-Viso recorder, model 150.†

The intensity (amplitude) of each contraction is measured by the rise in pressure (mm. Hg) it produces in the amniotic fluid. The frequency is expressed by the number of contractions per 10 min. The product (intensity × frequency) of the contractions is called uterine activity and is expressed in Montevideo Units (Mont. U.) A detailed description of the methods for the quantitative analysis of the records of amniotic fluid pressure has been published recently.[3] The tonus (lower pressure between the contractions) is also measured.

Methods for Studying the Coordination of the Contractions

The intramyometrial pressure is recorded simultaneously in 4 different parts of the uterus by means of 4 microballoons (volume: 0.02 ml.) inserted in the thickness of the uterine wall, each one connected to a recording electromanometer. This method enables us to study the origin of the contractile wave, its propagation through the uterus, and its force and duration in the different areas of the organ. Through these data, we may gather considerable information on the coordination of uterine action.[4, 5]

The Spontaneous Uterine Contractility During Normal Pregnancy and Labor

For a better understanding of the effects of oxytocin, it is necessary to know the spontaneous behavior of the uterus during the normal pregnancy cycle.

Pregnancy. The results obtained in more than 500 pregnant women studied are shown diagrammatically in FIGURE 1. During the first 30 weeks of pregnancy, uterine activity (striped area) is lower than 20 Mont. U. Tracing A illustrates the characteristics of uterine contractility at this time of pregnancy; very small contractions (which remain localized to a small area of the

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† Sanborn Company, Waltham, Mass.

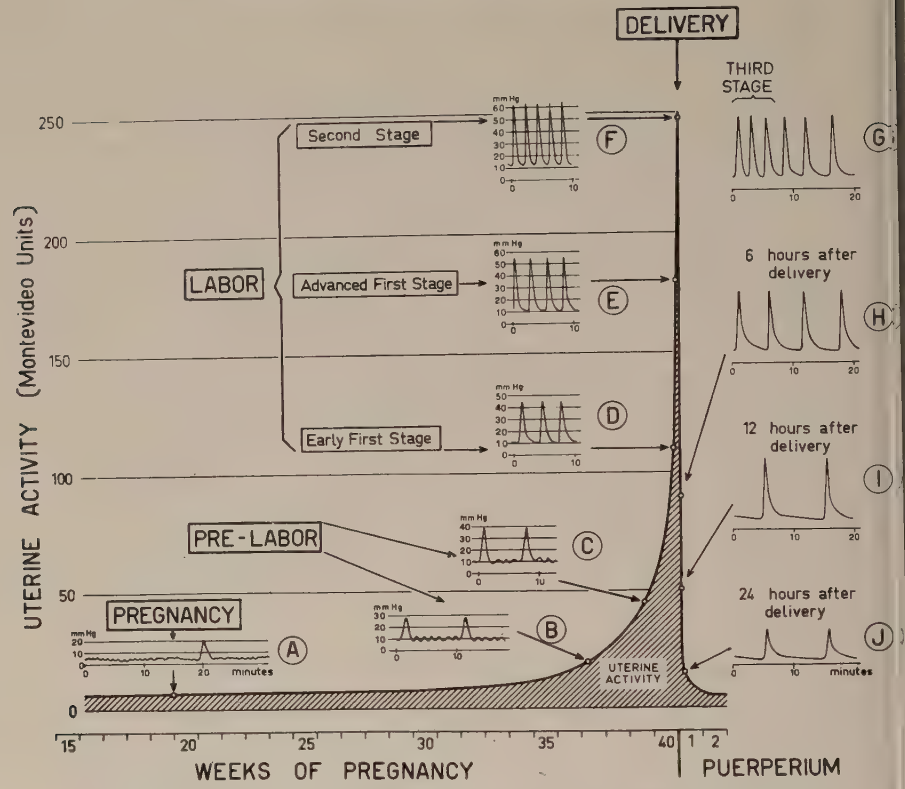

FIGURE 1. The evolution of spontaneous uterine activity throughout the pregnancy cycle—illustrated by the striped area and measured on the left-side scale. Typical (schematical) tracings of uterine contractility at different stages of the cycle are shown.

uterus) are produced every minute. From time to time a larger (Braxton Hicks) contraction is recorded.

Prelabor. After the thirtieth week, uterine activity increases slowly and gradually due to the increase of the intensity and frequency of the Braxton Hicks contractions (tracings B and C), which also improve their coordination, spreading through the uterus. This period of increasing uterine activity, lasting for several weeks, is called prelabor. During this period the uterine cervix is progressively ripened.

Beginning of labor. Uterine activity increases rapidly. Clinical labor usually starts when uterine activity reaches values between 80 and 120 Mont. U. (average 100 Mont. U.) There is no clear-cut division between prelabor and labor, only a gradual and progressive transition. The small localized and incoordinated contractions, typical of pregnancy, tend to disappear as prelabor advances and are absent from the tracings recorded in normal labor.

Labor. During labor, uterine activity continues to increase until the fetus is delivered. The contractions increase their intensity and frequency as shown by tracing D (early first stage), E (advanced first stage), and F (second stage of labor). In tracing F the intensity of the contractions is 50 mm. Hg, and their frequency is 5 contractions per 10 min.; uterine activity is equal to 50 × 5 = 250 Mont. U.

In normal labor uterine activity usually ranges between 100 and 250 Mont. U.

Early puerperium. After delivery of the fetus, uterine activity diminishes rapidly.* The frequency of the contractions usually decreases first, being reduced to 1 contraction per 10 min. (tracing I) 12 hours after the delivery. The intensity of the contractions diminishes afterward (tracing J).

THE UTERINE RESPONSE TO OXYTOCIN

Methodology

Oxytocin infusion. Natural pure oxytocin and synthetic oxytocin, supplied by du Vigneaud,[7] were used as standards of comparison. The majority of the experiments were carried on with synthetic oxytocin (Syntocinon).† No significant differences were observed between these preparations.[8]

Oxytocin was administered by continuous intravenous infusion, as advised by Theobald *et al.*,[9] Hellman,[10] and Page.[11] The infusions were made with a Continuous Slow Injector‡ provided with 6 speeds. The infusions were started at the rate of 1 milli Unit (mU.) of oxytocin per minute (1 mU./min.), which was maintained for 30 to 60 min.; the infusion rate was then increased to 2 mU./min. and maintained for a similar period of time. In this way the infusion rate was successively duplicated (4, 8, 16, 32 mU./min.) until uterine contractility reached values similar to those recorded in spontaneously beginning normal labor.

Clinical material. These infusions were carried on in 40 pregnant women from the seventeenth week of pregnancy onward. In 8 cases the uterine response to oxytocin was studied several times, in the same subject, at different stages of pregnancy.

The subjects were not in labor when the infusion started, although in some cases labor began during the study period as a consequence of the infusion (see below). We shall consider only the results attained before the onset of labor because, when labor starts, the stable conditions required for a pharmacological study no longer obtain.

Characteristics of the Oxytocin-Produced Contractions

If oxytocin is infused in the proper doses, it causes an increase of the intensity and frequency of uterine contractions without significantly raising the tonus (FIGURE 2);[3, 12, 13] it also accelerates the spreading of the contractile waves through the organ and improves the coordination of the contractions, which acquire the so-called Triple Descendent Gradient[5] characteristic of normal labor. In the tracings of amniotic fluid pressure and of intramyometrial pres- sure, the contractions produced by oxytocin infusion are indistinguishable from

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* The third stage of labor (tracing G) is accomplished by the first 2 or 3 contractions performed by the uterus after the delivery of the fetus.[6]

† Supplied by Sandoz Pharmaceuticals, Hanover, N. J.

‡ Manufactured by Palmer Ltd., London, England. A similar infusion pump can be obtained in the United States from The Harvard Apparatus Company Inc., Dover, Mass.

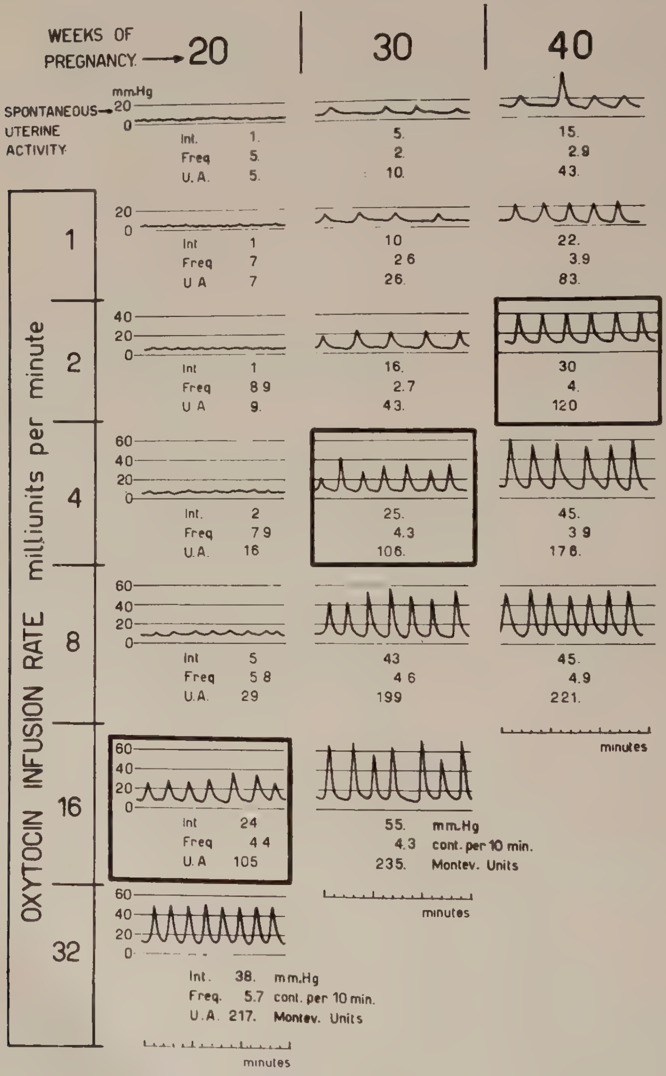

FIGURE 2. Uterine responses to oxytocin infusion obtained at the twentieth, thirtieth, and fortieth weeks of normal pregnancy. Typical segments of the original records of uterine contractility are shown. Each vertical column has been built with segments of the same record.

those recorded during spontaneously beginning, normal labor.* The physiological pattern of the contractions produced by the continuous intravenous infusion of oxytocin was recorded for the first time by Hellman *et al.*[14]

By increasing progressively the infusion rate of oxytocin, it is possible to reproduce at will all types of uterine activity spontaneously performed by the uterus at the different stages of prelabor and labor (compare FIGURES 1 and 2).

Induction of Labor

If the infusion of oxytocin is continued for the necessary length of time, labor will start and proceed normally. To start labor, it is necessary to produce a given amount of strong (40 mm. Hg) uterine contractions; this amount is large (between 200 and 300 contractions) when the uterine cervix is unripe, and it is small (less than 100 contractions) when the cervix is ripe.[12] In cases where it is not advisable to induce labor, the oxytocin infusion nevertheless can be performed, the only precaution being to keep its duration below a certain limit in order not to produce the amount of contractions needed for starting labor.

INFLUENCE OF THE GESTATIONAL AGE

Uterine Responses to Oxytocin Infusion at the Twentieth, Thirtieth, and Fortieth Weeks

FIGURE 2 shows tracings of uterine contractility obtained in the same subject at the twentieth, thirtieth and fortieth weeks of pregnancy (respectively, in the vertical columns on the left side, in the middle, and on the right side).

The upper row shows the spontaneous activity of the uterus at the three stages of pregnancy. The underlying rows show the effects of the infusion of oxytocin; each row corresponds to a given infusion rate (indicated on the scale at the left side).

The black frames indicate (for each gestational age) the row where uterine activity has reached labor ranges (over 100 Mont. U.) At the fortieth week, 2 mU./min. are enough to raise uterine activity over 100 Mont. U.; at the thirtieth week, 4 mU./min. are required and, at the twentieth week, 16 mU./min.

At the twentieth week the spontaneous activity is very low and infusion rates under 8 mU./min. are almost without effect on the uterus; however, when the infusion rate is increased more than 8 mU./min., uterine activity is raised to the labor range of values. With 32 mU./min., uterine activity becomes similar to that recorded in advanced stages of labor. Roughly, it can be stated that, in order to obtain similar uterine responses, the infusion rate must be 8 times higher at the twentieth week than at term pregnancies.

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* The small, localized and incoordinated contractions recorded during pregnancy and during abnormal labor are seldom seen in the records obtained during oxytocin infusions, which usually show only the strong, rhythmic, and well-coordinated contractions characteristic of normal labor.

At the thirtieth week the spontaneous activity is slightly higher than at the twentieth week, but the response to oxytocin has increased greatly: 1 and 2 mU./min. producing definite effects and 4 mU./min. sufficing to raise uterine contractility to labor values.

At the fortieth week the spontaneous activity is much higher than at the thirtieth week and uterine response to oxytocin has also increased, but in a smaller degree than between the twentieth and thirtieth weeks.

Evolution Throughout Pregnancy of Uterine Response to Oxytocin Infusion

Figure 3 shows the results obtained from 6 records made at different stages of pregnancy. Three of these 6 records are those shown in figure 2. The 6 records were selected from a total of 62 because they were considered typical for their respective gestational ages.

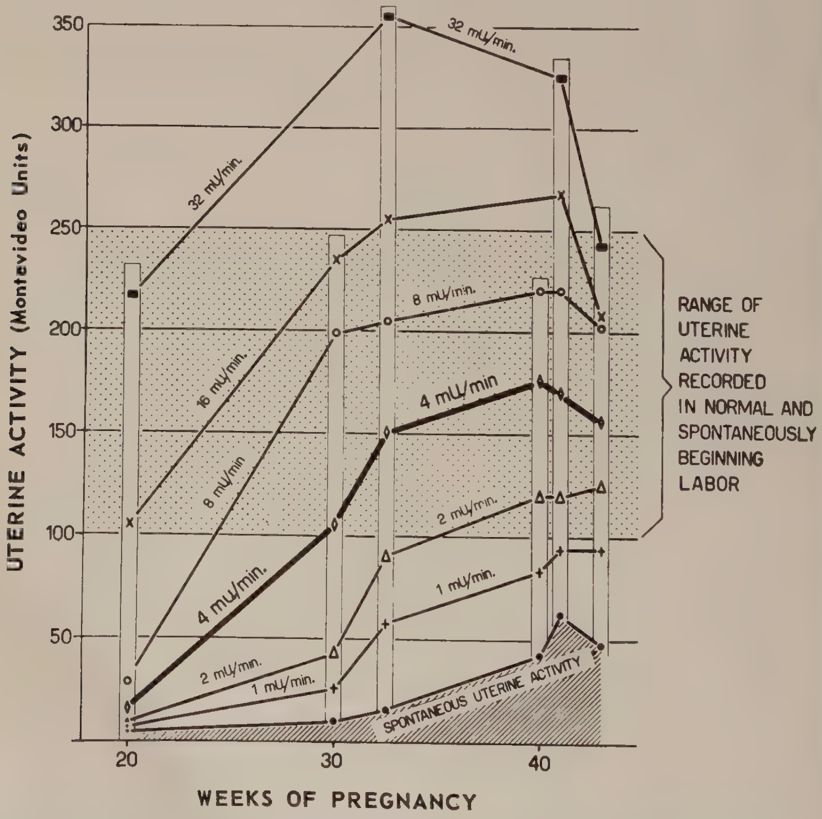

Figure 3. Evolution of the uterine response to oxytocin infusion during the second half of pregnancy. Each vertical column corresponds to one record. The thick line that joins the rhombus indicates the evolution of uterine response to 4 mU./min. The other lines correspond to the other infusion rates.

The spontaneous uterine activity (striped area) follows the same general course as indicated in FIGURE 1, the only difference being that, in term pregnancies, and as the subjects considered in FIGURE 3 were not in labor, the spontaneous activity remains well below the labor range of values, not surpassing 60 Mont. U.

The uterine response to low infusion rates (1 and 2 mU./min.) increases slowly between the twentieth and thirtieth weeks; it increases rapidly after the thirtieth week, and again continues to increase very slowly during the last weeks of pregnancy. The response to high infusion rates (8 and 16 mU./min.) increases sharply between the twentieth and thirtieth weeks, and then increases very little until term. The response to medium infusion rates (4 mU./min.) follows an evolution halfway between the other two and is considered as the most representative for illustrating the changes of uterine response to oxytocin infusion during pregnancy.

Uterine response to oxytocin infusion increases with the age of pregnancy, reaching almost its maximum values at the thirty-sixth week, and showing little changes between the thirty-sixth and the fortieth weeks and during labor. Theobald *et al.*,[15] Aburel *et al.*,[16] and Smyth[17] state that "uterine irritability" increases greatly during the days preceding labor, a concept currently accepted among obstetricians and physiologists. However, these authors fail to fulfill one or more of the following requirements: (1) accurate recording of uterine contractions; (2) quantitative evaluation of the spontaneous uterine activity; and (3) administration of oxytocin by continuous intravenous infusion.

In prolonged pregnancies (that is, after the fortieth week) the spontaneous uterine activity and the response to low infusion rates are similar to those recorded at the fortieth week; high infusion rates produce uterine responses smaller than those obtained at the fortieth week.

At each one of the different stages of pregnancy considered in this paper, the uterine activity produced by very high infusion rates (32 mU./min.) is very close to the maximum activity that can be performed by the uterus. Already at the twentieth week the infusion of 32 mU./min. produces a strong activity (comparable to that of advanced labor); the maximum uterine response increases until some time after the thirtieth week, and then decreases slightly during the last weeks of pregnancy.

Differences Between the Rabbit and the Woman in the Uterine Response to Oxytocin Throughout Pregnancy

In the rabbit (according to Schofield[18]), from the second to the twenty-ninth days of pregnancy, the uterus does not respond at all to oxytocin (FIGURE 4), even if administered at very large doses (I.V. injection of 5,000 mU.). In the last 2 or 3 days of pregnancy the uterus reacquires its reactivity to oxytocin, the average threshold dose being 24 mU. of oxytocin. Thus, a very great increase occurs in the uterine response to oxytocin during the stages of pregnancy just preceding labor.

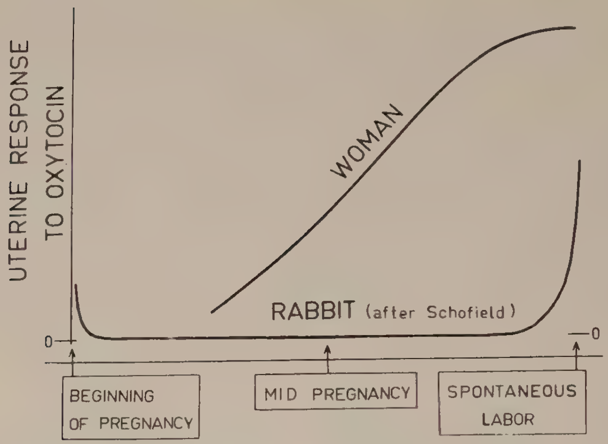

FIGURE 4. Differences between rabbit and woman in the evolution of uterine response to oxytocin throughout pregnancy (highly schematic).

In contrast, the pregnant human uterus always responds to oxytocin;* the important increase of that response occurs before the thirty-sixth week. No significant changes are observed during the last 4 weeks of pregnancy or during labor.

THE DOSE-EFFECT RELATIONSHIP

Influence of the Gestational Age on the Dose-Effect Curves

FIGURE 5 shows, for the same three records of FIGURE 2, the uterine activity plotted against the infusion rate of oxytocin (dose-effect curves). Each curve starts (on the ordinate axis) from the value of the spontaneous uterine activity (black circles), which increases with the gestational age.

The influence of gestational age can be appreciated easily. At the twentieth week the dose-effect curve is very flat, indicating the small uterine response to low and medium infusion rates; the curve rises when high infusion rates are reached. At the thirtieth week the curve is much steeper, showing the increase in uterine response to low infusion rates. The increase in slope of the curve is still more evident at the fortieth week.

Shape of the Dose-Effect Curve

Every dose-effect curve rises asymptotically to a maximum value of uterine activity, which ranges between 250 and 350 Mont. U.

The curves corresponding to the twentieth and thirtieth weeks are S shaped, as should be expected for a curve relating the concentration of a drug and its effects.†

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* Our earliest studies have been performed at the seventeenth week of pregnancy, but Moir[19] has tested the uterus at the ninth week of pregnancy and found it responsive to oxytocin.

† We assume that the concentration of exogenous oxytocin in the blood is proportional—for a given experiment—to the rate of infusion of the drug.

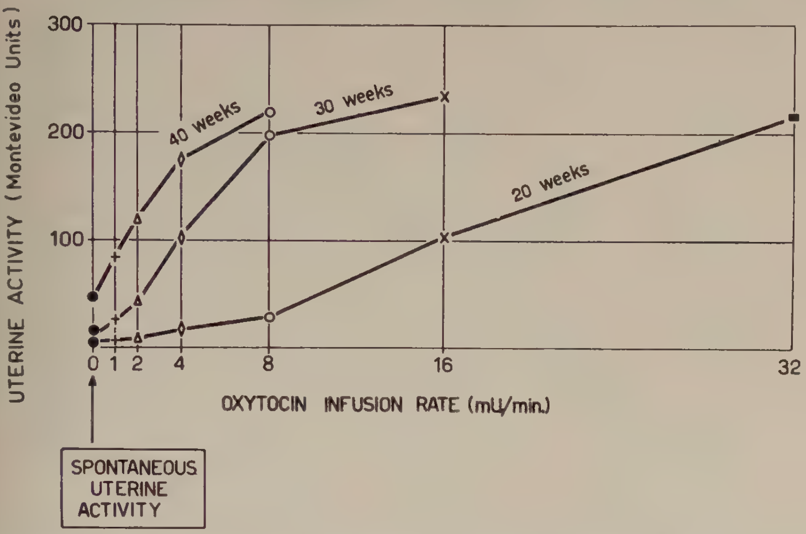

FIGURE 5. Dose-effect curves at the twentieth, thirtieth, and fortieth weeks of normal pregnancy. Each curve corresponds to one of the records shown in FIGURE 2. The spontaneous uterine activity of each record is indicated by black circles. The uterine activities recorded during the different infusion rates of oxytocin are indicated with the same symbols as in FIGURE 3.

The curve corresponding to term pregnancy also can be considered as S shaped, if we assume that the lower part of the S is placed at the left of the axis of the ordinates (due to the increase of the spontaneous activity and of the slope of the curve).

Dose-Effect Mathematical Relationship During Late Pregnancy

FIGURE 6 shows the mean dose-effect curve of 20 cases studied between the thirty-sixth and the fortieth weeks of normal pregnancy. During these 4 weeks, uterine response to oxytocin is very consistent and shows little variation, as illustrated by the Standard Deviation of the Mean curve of FIGURE 6.

The function of this mean curve[3] is

$$UA = UA_{\text{spont}} + \Delta UA_{\max}(1 - e^{-R.\text{inf}})$$

where UA is the uterine activity recorded during the oxytocin infusion; inf is the rate of the infusion; UA_{spont} is the spontaneous uterine activity existing before the infusion is started; $\Delta UA_{\max}$ is the maximum increase of uterine activity (over UA_{spont}) that will be produced when inf increases infinitely; and R measures the reactivity of the pregnant woman to oxytocin infusion and depends upon (1) the sensitiveness of the myometrium to oxytocin, and (2) the "clearance" of oxytocin. These two factors will be discussed below.

ABNORMAL RESPONSE TO OXYTOCIN

Response higher than normal. In toxemia of pregnancy,[12] the spontaneous uterine contractility and the response to oxytocin infusion are much higher

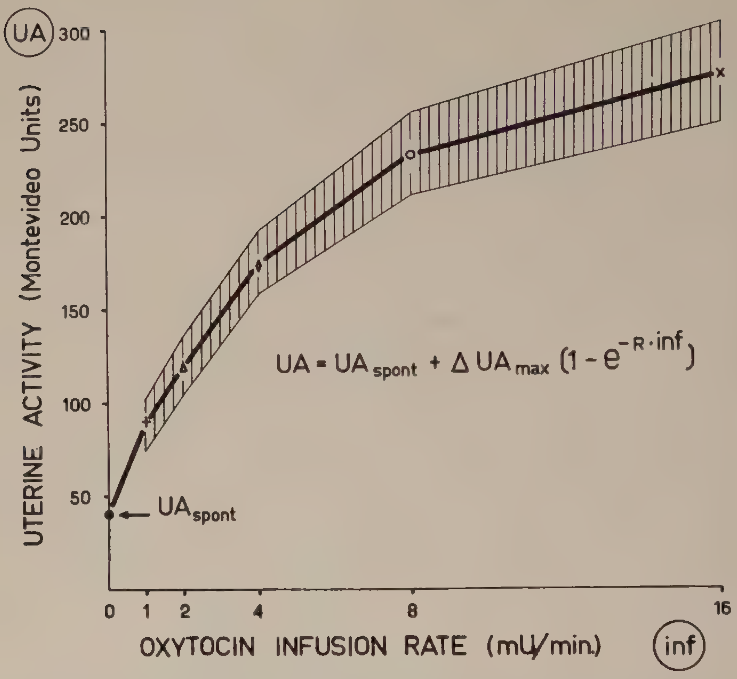

FIGURE 6. Mean dose-effect curve at late normal pregnancy (between the thirty-sixth and fortieth weeks). Average of 20 cases. The striped area indicates ±2 Standard Deviations

than normal, as illustrated in FIGURE 7 by the dose-effect curve corresponding to a case of eclampsia. These findings confirm previous reports.[20–22]

Responses lower than normal. These have been found in polyhydramnios,[2] myxedema,[24] and elderly primigravida[12] (FIGURE 6), and in 2 cases that were clinically considered as normal pregnancies. In 7 cases with fetal death,[12] the response was normal in 6 cases and lower than normal in 1 case.

POSTINFUSION DISAPPEARANCE OF OXYTOCIN EFFECTS

After the interruption of oxytocin infusion (FIGURE 8), uterine activity diminishes progressively. If labor has not started, uterine activity returns to the original preinfusion level of spontaneous activity. If labor has already started, uterine activity decreases after interrupting the infusion, but remains stabilized at a higher level of spontaneous activity;[12] this level increases as labor advances.

In mid-pregnancy the decline in uterine activity is very rapid, being completed in 15 to 25 min. The decline becomes progressively slower until the thirty-sixth week, when approximately 1 hour is required in order to return to the spontaneous activity level. No changes are observed in the rate of decline between the thirty-sixth and fortieth weeks.[25]

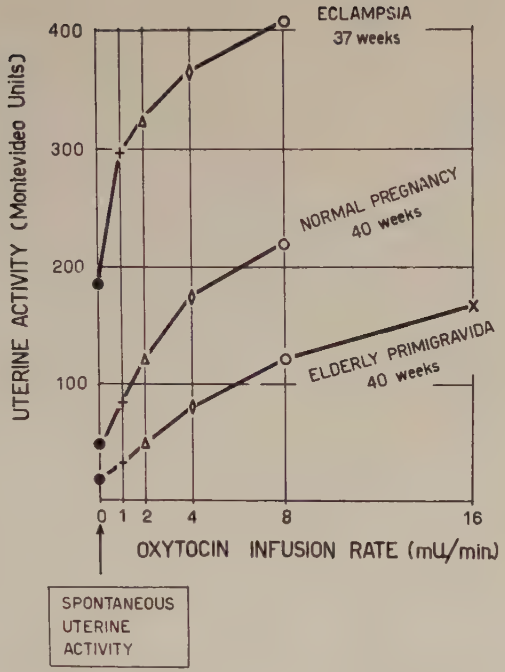

FIGURE 7. Dose-effect curves in abnormal cases, where the uterine response to oxytocin infusion and the spontaneous activity are much higher—as in eclampsia—or significantly lower—as in a case of an elderly primigravida—than in normal term pregnancies.

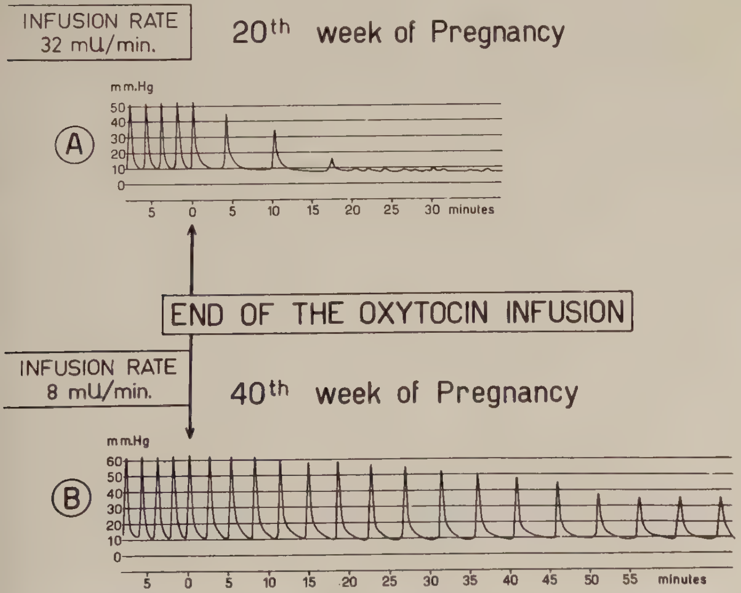

FIGURE 8. After the interruption of oxytocin infusion, uterine activity diminishes much more rapidly at midpregnancy (A) than at term pregnancy (B).

These results are in disagreement with those of Page,[11] who claims that in late pregnancies the uterine effects of oxytocin disappear 3 min. after the infusion is discontinued.

Discussion

The Increase of Uterine Response to Oxytocin Infusion Occurring as Pregnancy Advances

Two mechanisms can explain this increase (FIGURE 9):

(1) *Augmentation of the sensitiveness of the myometrium.* The response of the uterus to a given concentration of oxytocin in the internal environment increases throughout pregnancy.[26]

(2) *Reduction of the total ability of the body to inactivate and excrete oxytocin ("clearance" of oxytocin).* The lower the clearance, the greater the blood level of oxytocin (and consequently the uterine response) produced by a given infusion rate of oxytocin.

The rate of decline of uterine activity (after the interruption of the oxytocin infusion) can be used as an indirect measure of the clearance of oxytocin in the body (FIGURE 8). If this assumption is correct, the clearance of oxytocin would be high in the twentieth week and would diminish until the thirty-sixth week, then remaining unchanged (FIGURE 10).[15] These changes of the clearance could be partially responsible for the opposite simultaneous changes occurring in the uterine response to oxytocin infusion (FIGURE 3).

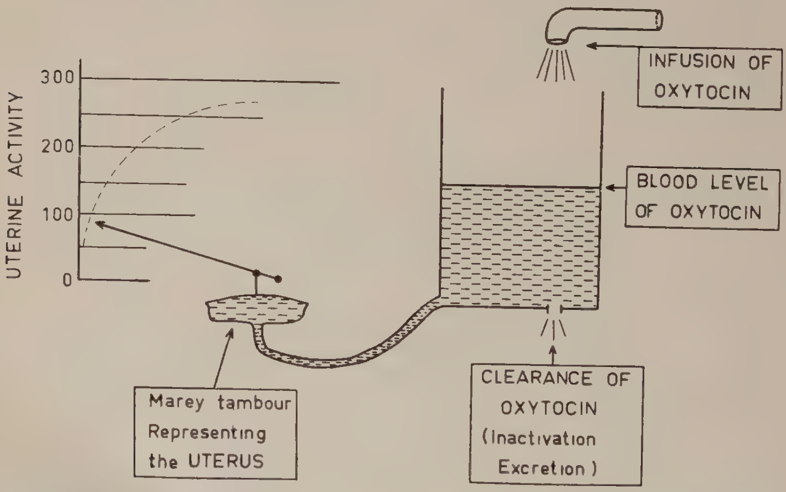
300
200
100
0
UTERINE ACTIVITY
INFUSION OF OXYTOCIN
BLOOD LEVEL OF OXYTOCIN
Marey tambour Representing the UTERUS
CLEARANCE OF OXYTOCIN (Inactivation Excretion)

FIGURE 9. Hydraulic model illustrating two factors conditioning the response of the uterus to the infusion of oxytocin (highly schematic). These factors are: (1) the sensitiveness of the myometrium (Marey tambour) to the oxytocin of the blood (hydrostatic pressure of water in the container); (2) the "clearance" of oxytocin, that is, the total ability of the pregnant woman to inactivate and excrete oxytocin, indicated by the area of the hole in the bottom of the container.

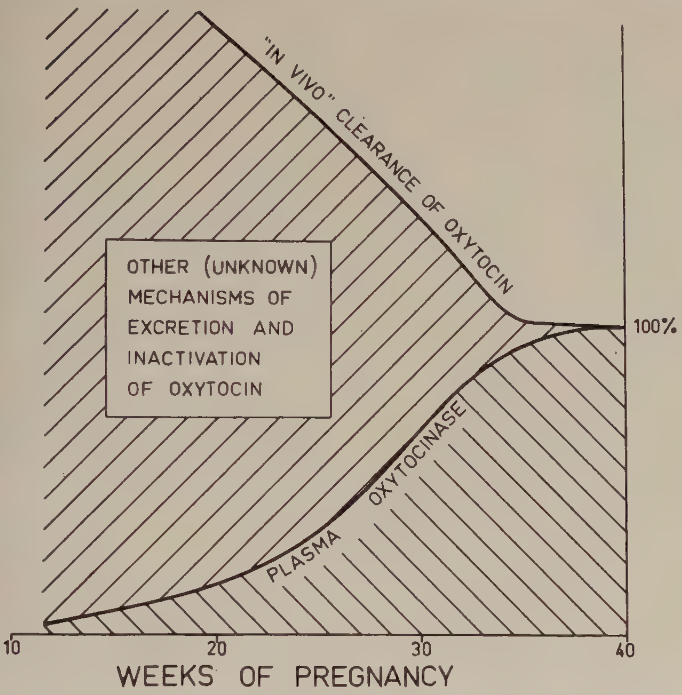

FIGURE 10. Comparison between the evolution throughout pregnancy of the *in vivo* "clearance" of oxytocin and of the plasma oxytocinase (highly schematic). The values found at term pregnancy have been adopted as 100 per cent.

The Mechanisms of the Clearance of Oxytocin Throughout Pregnancy

In the woman, the ability of plasma to inactivate oxytocin* increases markedly as pregnancy advances,[27-31] reaching almost maximum values at the thirty-sixth week[32] and showing little change until the end of pregnancy (FIGURE 10).† This increase is due to an enzyme, the oxytocinase,[34] which seems to be produced by the placenta.[35, 36] Oxytocinase appears in the blood only during pregnancy, and only in those species with placenta hemochorialis (human and monkey).

During the last 4 weeks of pregnancy the blood plasma incubated at 38° C. with oxytocin requires 7 to 9 min. to inactivate 50 per cent of the added oxytocin.[32] These *in vitro* results are in close agreement with those obtained *in vivo* by Sica-Blanco and González-Panizza,[26] who have estimated that 9 min. are required for the disappearance of 50 per cent of the oxytocin existing in

* In the inactivation by plasma oxytocinase, no significant difference has been observed[32] between synthetic oxytocin (Syntocinon) and natural oxytocin (Pitocin), as has been claimed by Semm.[31]

† Dicker and Tyler[33] are the only authors who claim that the ability of the plasma to inactivate oxytocin decreases as labor approaches and is abolished in the plasma obtained during normal labor.

the body of the pregnant woman at the moment of the interruption of the oxytocin infusion.* It follows that, during the last 4 weeks of pregnancy, plasma oxytocinase may account (as the only acting mechanism) for the *in vivo* clearance of oxytocin (FIGURE 10).

In mid-pregnancy, on the contrary, plasma oxytocinase, which is much lower than during late pregnancy,[32] cannot explain by itself the high *in vivo* clearance of oxytocin existing at that time.[25] This discordance suggests that other mechanisms of clearance (inactivation or excretion) of oxytocin must be active in mid-pregnancy (FIGURE 10).

A great diminution of these unknown mechanisms (such as, renal excretion and hepatic inactivation) must be postulated in order to explain the lowering of the *in vivo* clearance of oxytocin that occurs as pregnancy advances,[25] in spite of the simultaneous increase of plasma oxytocinase (FIGURE 10).[32] It should be emphasized that the *in vivo* estimation of oxytocin clearance[25] and the *in vitro* determination of the plasma oxytocinase[32] have been carried on in the same group of pregnant women.

Estimation of the Rate of Secretion of Oxytocin that Would be Necessary for Producing Labor

Assuming that, in the pregnant woman, endogenous oxytocin is the principal factor that increases uterine activity during prelabor and labor (see below), there are two possible mechanisms that could explain this action (1) an increase of the uterine response to a constant secretion rate of oxytocin and (2) an augmentation of the rate of secretion of oxytocin.

We have seen (FIGURE 3) that in the pregnant woman there are no important changes in the uterine response to oxytocin infusion, neither between the thirty-sixth and the fortieth weeks of pregnancy nor during labor. Therefore it is necessary to postulate an augmentation of the rate of secretion of oxytocin in order to explain the great increase of uterine activity that occurs during spontaneous prelabor and labor. At the beginning of labor (uterine activity: 100 Mont. U.) the secretion rate† should be in the neighborhood of 2 mU./min. (FIGURE 11); and it should increase up to 8 mU./min. in order to raise uterine activity to the over-200 Mont. U. usually recorded during advanced labor.

The progressive distension of the uterine cervix and of the vagina that occur as labor advances could be one of the causes that reflexly increase the secretion of oxytocin during labor.[37] After delivery of the fetus, as the distension of the birth canal ceases, the oxytocin secretion and the uterine activity diminish very rapidly (FIGURE 1).

Is Oxytocin the Hormone that Produces Spontaneous Labor in the Woman?

A complete discussion of the subject is not possible here; for this purpose, the reader is referred to Cross,[38] Harris,[39] Fitzpatrick,[40] and Theobald *et al.*[15]

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* These estimates are in disagreement with those of Page, who states that only 50[27] or 90[11] sec. are needed for the inactivation *in vivo* of 50 per cent of the oxytocin by the action of "pitocinase" (oxytocinase).

† Assuming that the secretion of endogenous oxytocin has the same uterine effects as the infusion of exogenous oxytocin made at a similar rate.

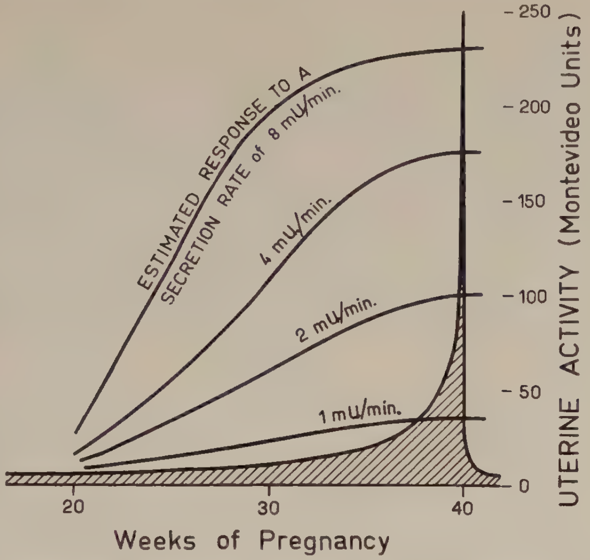

FIGURE 11. Comparison between the recorded values of spontaneous uterine activity during pregnancy, prelabor, and labor (striped area) taken from FIGURE 1 and the estimated uterine responses to several rates of secretion of endogenous oxytocin (highly schematic).

We shall present only some facts taken from our own research in the pregnant woman which speak in favor of the oxytocin hypothesis of labor.

(1) All the characteristics (shape, coordination, intensity, frequency, duration, and tonus) of the contractions produced by the infusion of oxytocin on the pregnant human uterus are indistinguishable from those of the contractions recorded in spontaneous labor.

(2) The doses of oxytocin necessary to inject per minute per kilogram of body weight, in late pregnancy, in order to raise uterine activity to labor values, are of the same general range as the doses of ADH (the twin sister of oxytocin) required for the inhibition of water diuresis.[39, 41] This fact was first noticed by Theobald *et al.*[9]

(3) The infusion of oxytocin, lasting for from 5 to 10 hours, causes a significant increase of plasma oxytocinase.[32] The similar increase that occurs spontaneously during pregnancy can be accepted as indirect evidence of an augmented secretion of endogenous oxytocin.*

(4) We have recorded significant increases of the contractility of the pregnant human uterus as a consequence of the action of the following agents [which are known to cause the release of oxytocin and antidiuretic hormone (ADH)]:[19, 37, 39, 42-44] (a) intravenous injection of hypertonic NaCl, morphine,[45]

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* Samuels,[48] speaking about ADH, says that "the rate of destruction of the hormone indicates its importance in relation to immediate adjustments of the environment." Applying this view to oxytocin, we can consider that the augmentation of the ability of the plasma to inactivate the hormone, which occurs as pregnancy advances, suggests that oxytocin has an important role in parturition.

and pyrogenic agents (with production of chills); and (b) stimulation of the nipples or distension of the uterine cervix or vagina.

(5) It has been estimated in our laboratory[26] that, during labor induced by oxytocin infusion, the concentration of exogenous oxytocin in blood is between 20 and 40 mU. per liter of plasma.* These figures are in good agreement with those presented by Theobald *et al.*[9, 15] and by Fitzpatrick,[40] which have been obtained by different methods.

If the blood concentration of endogenous oxytocin during spontaneous labor were of the same range, it would be difficult to make its determination with the methods available at present.† The surprisingly high concentration of endogenous oxytocin found by Bisset *et al.*[47] in the blood of humans could be due to the anesthetic employed during the determination.

SUMMARY

A quantitative study of the contractility of the pregnant human uterus is made by the recording of the amniotic fluid pressure.

The uterine response to the continuous intravenous infusion of oxytocin increases with the age of pregnancy, reaches almost its maximum values at the thirty-sixth week, and shows no significant changes between the thirty-sixth and fortieth weeks and during labor.

In term pregnancies, infusion rates between 2 and 8 mU. of oxytocin per minute are sufficient to raise uterine activity to the lowest and highest values, respectively, recorded during spontaneous normal labor. At the twentieth week, infusion rates 8 times higher are necessary for the same purpose.

For each stage of normal pregnancy there is a consistent quantitative relationship between the response of the uterus and the infusion rate of oxytocin. In toxemia of pregnancy, uterine response is higher than normal.

The mechanisms conditioning the uterine response to the infusion of oxytocin, and the *in vivo* "clearance" of the injected oxytocin are discussed. The role of endogenous oxytocin in spontaneous normal labor is discussed also, and its hypothetical secretion rate is estimated.

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* These estimations were based on the assumption that all the oxytocin was contained in the intravascular compartment; if oxytocin diffuses to the interstitial fluid, its concentration should be much lower. Heller[46] has shown that synthetic oxytocin binds with plasma proteins.

† The same conclusion has been reached by Heller[46] in relation to the concentration of endogenous ADH in peripheral blood of man and rat.

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Part VII. Menstruation and Menstrual Disorders

A THEORY OF MENSTRUATION

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A worthwhile theory rests on accepted facts proved within contemporary accuracy. One wishing to theorize, then, on the causes and nature of menstruation utilizes pertinent data that predecessors or colleagues have apparently verified.[1] In order to complete a theory, one must use a few assumptions based on what one or another believes will later prove to be facts. Indeed, if none such is needed, the concept transcends theory and is itself factual. The reader is exhorted to consider as mere working hypotheses much herein that reads like statement of fact.

Hormonal Agents Involved in the Reproductive Cycle

We think of menstruation as the result of the interplay of a few familiar hormones. Hence we shall briefly review their respective roles, individually, synergistically, and reciprocally, not only in the sterile cycle, of which menstruation is the end point, but also in the so-called "conceptive" cycle. For, as has been succinctly expressed by Bartelmez:[2] "The distinctive events of the menstrual cycle can be interpreted only in the light of the early stages of pregnancy."

(1) *Estrogen.* This is the name of a hormonal "genus," comprising, among other "species" that do not concern us, at least two that do, namely: the type-steroid, estrone, and also estradiol, which was long regarded as the mother-secretion of the follicular complex. These proliferate the endometrium from the simple comparatively inactive mucosa that is typical of the castrate into an intermediate working tissue producing first a secretion probably specialized for spermatozoal purposes. This is squeezed into the uterine cavity by frequent intermittent myometrial contractions themselves also mediated by estrogen.

(2) *Progesterone.* This is elaborated by the corpus luteum. It is a particular steroid of the "genus" first named by Corner[3] and Allen progestin, and now, for a good reason, often called gestogen. It evokes, with the help of estrogen, such changes in the endometrium and myometrium, too, as will fulfill the requirements of the conceptus.

(3) *Gonadotropin.** This generic term is applied to a glycoprotein[4] manifesting three hormonal effects. It may comprise three distinct agents that, however, are so similar chemically as to justify the suspicion that they are but three variants of the same substance.[5] They come from the anterior hypophysis, probably in response to the neurohumoral activity of the hypothalamus,[6] and each is thought to change in a particular manner the separate components of the follicular apparatus.[7-9] The three gonadotropins that evoke changes are:

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* Webster defines *tropic*, with only a *p*, as "tending to change"; *trophic*, with a *ph*, as "to nourish." The hypophyseal product does more than nourish; it changes.

(a) *Follicle-Stimulating Hormone, FSH.* This transforms (probably with major assistance from the next variant, LH) one of the resting intermediate ovarian follicles into the mature Graafian follicle.[10] From this, issue increasing amounts of estrogen and gradually more easily recognizable modica of progesterone.

(b) *Luteinizing Hormone, LH (also Known as Interstitial-Cell-Stimulating Hormone, ICSH).* This changes the internal theca of the follicular complex from a mere fibrous sheath into a glandular ("luteinized") structure whence emanates not only estrogen but also preovulatory progesterone. After ovulation, hormone-production is localized mainly in the luteinized granulosal layer.[11]

(c) *Luteotropic Hormone, LtH (also Known as Prolactin, or Lactogenic Hormone).* This probably substantiates the granulosa as a ductless gland,[7-9, 12, 13] and may possibly help in the mysterious changes in the enzymatic condition of one tiny thinned-out portion of the follicular wall so that it ruptures. Through the opening, escape the ovum and its surrounding cumulus, as well as fragments of granulosa. For about two weeks, LtH sustains the remaining granulosa as a secretory gland which contributes to the blood stream increasing amounts of both progestrone and estrogen. While regressing, this body gradually turns yellow (becomes luteolus); hence the name corpus luteum.

As stated, estrogen and progesterone work together on the endometrium and on the myometrium. Together, they order the progestational functioning of these tissues. The prior dominance of estrogen gives way to that of progesterone during the three or four days of the so-called "ovulation phase," which begins as follicular rupture approaches and ends with the establishment of the corpus luteum. The succeeding dominance of progesterone over estrogen, in terms of endometrial results, is neutralized when, after about two weeks, completed subsidence of the corpus luteum[14] critically reduces the supply of both ovarian hormones. The endometrium, unable to maintain itself without them, regresses and disrupts.[15-20] This results in the process on which we theorize: menstruation.

The stated sequence of action of the two ovarian hormones is doubtless due to coordinate alternations in primacy of the gonadotropic roles of the versatile anterior hypophysis. It seems increasingly likely that estrogen, in addition to its effects on the uterus, exerts, when in appropriate hematic concentration, a weakening of FSH's reign within the ovary and doubtless encourages the pituitary to increase LH production.[10]

Weakly subordinate at first, LH intrudes into a duality of effectiveness on the maturing follicles. This raises progesterone output to cooperate with estrogen, which also increases. When both are in action, the adenohypophysis reacts by secreting the gonadotropin-variant called LtH. This, in turn, while not interfering with the gradual increase of estrogen, may somewhat abruptly invigorate the corpus luteum's progesterone output. At all events, potentiation persists during most of the fortnightly postovulatory phase.

The ability of the pituitary gland to produce the follicle-stimulating and two luteinizing hormones gradually and somewhat enigmatically wanes after the complete ascendancy of progesterone influence on the endometrium has been established. This is perhaps by inhibitory action of progesterone or of the combined ovarian steriods. Unless these particular tropic faculties of the hypophysis can be replaced by the only stimulant of which we are aware, namely the chorion-hormone from the imbedded trophoblastic syncytium,[21] the corpus luteum breaks down and the quantity of estrogen-progesterone rapidly diminishes. Hence the maintenance of the changes that these two hormones wrought in the uterus, purely in order to facilitate nidation, can no longer prevail. The endometrium must regress to a lower status, to a condition that can be maintained by the much diminished estrogen from the as yet immature follicles. Their delivery of this estrogen is, in turn, dependent on much reduced, but yet effective, pituitary gonadotropin.

We acknowledge our complete indebtedness to Bartelmez,[22-26] Markee,[27, 28] and Ramsey,[29, 30] as well as to Hisaw[17, 20] and Corner.[1, 15, 19] Significant knowledge has also been contributed by Daron,[31] Phelps,[32] and Hartman.[16, 18] To all of them we are indebted for delineation of the specific modifications in form and function accomplished for the conceptus in the endometrium by the ovarian steroids, separately and jointly. Particularly from the first two authors mentioned, we learn of the retreat of the hormonally starved endometrium when conception fails and there is no chorion-hormone to prevent the regression of the corpus luteum. This endometrial retrenchment is accomplished partly with preserved integrity and partly with the disorganization that produces menstruation.

Provision for the Nutrition of the Embyro

Quite obscure, except for fluid, are the requirements of the human morula when, at three to four days of age, it emerges from the oviduct into the uterus.[33] As cleavage of the blastomeres progresses, differentiation selects a few of them eventually to evolve the embryo; others, the placenta. The former group remains within a protective vesicle bounded by the others, their auxiliaries. Altogether they constitute the blastocyst. The slightly increased bulk of total protoplasm within both embryo and envelope during the ovum's early sojourn in the uterus suggests the incorporation of building material from the surrounding fluid medium. Probably to supply needed mucopolysaccharides, glycogen,[34] and other nutrients, the mucosa of the uterus is perforated by the orifices of tubules lined by secretory epithelium that extend outward from their basal origin.

During this phase there also begins the process that is the most necessary of all if the endometrium is to fulfill its major function of furnishing the trophoblastic conceptus with its indispensable requirement, maternal blood. To this end, estrogen causes active growth and extension toward the surface of those very special infrequently branched arteries, each of which is to take care of its narrow longitudinal segment of mucosa. Because even in the early preovulatory, estrogenic, phase they course upward in a screw-like manner and later in multiple twists and loops, they are called coiled or spiral arteries.[31] Reaching its own subepithelial area, the branches of each of these coiled arteries fan out into capillaries that intermingle, here and there, with those in neighboring fields. Thus all the surface is insured of trophoblast-essential blood. Contemporary and somewhat similar extension upward of the venous system occurs. By the time ovulation is imminent, the previously increasing amounts of estrogen and of the then subordinate progesterone have brought the endometrium back to its full thickness. The stroma is amply supplied with a colloidal ground substance and is in some places even moderately edematous. The nuclei of stromal cells have increased somewhat in size, but the cytoplasm still consists largely of ramifying tendrils that constitute, with some collagen fibers, the reticulum of the tissue. Through this, the arterioles coil upward to end in their loose capillary network. As postulated, for timely service to aspiring spermatozoa but surely in dutiful expectation of the kingly (or queenly) morula, the segmented ribboning glands, also by virtue of plentiful estrogen and some progesterone, are placing their secretion of glycogen and mucin[34] within their lumina. This the frequent and ever strengthening contractions of the myometrium force into the uterine chamber for use by the four- to five-day old blastocyst.

During only twenty-four to forty-eight hours, however, much more must be accomplished by the endometrium and quickly; for, when only five and one half to six days old, the rapidly growing and differentiating conceptus has probably begun to imbed.[35] Now it must achieve access to maternal blood or perish. Fortunately—reacting to high estrogen and rapidly increasing progesterone from the emergent corpus luteum—the coiled arteries proliferate and gradually add to their spirals, for the stroma does not stretch commensurately. Quite as important is the elaboration of the terminal capillary net of these vessels. This multitude of tiny proximate capillaries is there to gratify the assiduous foraging syncytium. For a day or two this, at first, ruthlessly phagocytizes the uterine epithelium lying between glandular orifices. It then attacks the thinly spread intervening stromal cells.[36, 37] Among them, it eventually strikes "pay-dirt" within a capillary, easily found and breached.

By the ninth day after ovulation, the "early primordium of the uteroplacental circulation" is present[37] and, within the next few days, the syncytiotrophoblast spreads outward rapidly into the stroma and is forming an internal sponge-like lacunal labyrinth into which, by the end of the second week of gestation, maternal blood will flow easily. Stimulated and supported by high estrogen and especially by the now dominant progesterone, the subcapillary coiled arterioles arise to this contingency and, individually or through their anastomosed branches, if they have them, expand in many endometrial segments into arteriolar sinusoids.

The older, well-developed arterial walls are sturdy and have an intrinsic intermittent contractility. The generously augmented amount of blood freely pouring outward through them from the basal arteries might expose the fresh arteriolar terminal walls or even the capillaries to a maternal systolic pressure that would rupture them and wash off the flooded conceptus. This is guarded against by the many large and small spirals, supplemented by the intermittent contractions.[25]

Progesterone brings first an increase in stromal ground substance. As the new corpus luteum accelerates in function, depolymerization liquefies this material, giving a more generalized edematous appearance to the tissue.[38] Finally, probably to buttress the all-essential arterial system, the watery reticulated ground substance is drawn within the outer-zonal stromal cells, first around the arterioles and within the capillary net. These stromal cells approximate as, more and more, they increase in size and extent. Eventually, toward the end of the second week of the luteal phase, they form a firm predecidual matrix within which the vessels lie well protected.

Meanwhile the glandular epithelial cells continue to empty themselves of a perhaps modified secretion. This in the lumina may seem of greater bulk because, as progesterone weakens myometrial activity,[39] the secretion more or less stagnates.[24] Therefore it dilates the glands. It is barely possible that the progesterone-dominated secretion is specially constituted and retained as nourishment for the trophoblastic vesicle and its embryo, since the marauding syncytium sometimes breaks through the epithelium and permits contact, at least, of this mucus with lacunar content.

The intense dedication to the ovum's welfare of the trophic ovarian progestational hormones, through their target endometrial structures, continues the proliferation and functioning described above. This has begun just after a previous sterile cycle but persists for several days after the hypersensitive hypophysis diminishes its support of the corpus luteum.[14] The resultant decline in ovarian steroids, quite slow at first, is felt by the endometrium only after about five more days of futile further expansion to conserve a nonexistent embryo. The afore-mentioned retreat is then promptly instituted near the twelfth postovulatory day, although the hypophyseally dependent corpus luteum has reached its zenith about seven days after follicular rupture.

Menstruation

The foregoing discussion displays the structure which, when nidation fails, menstruates, as well as the hormonal factors that have built it up. Without the continued support of these factors, the structure could not maintain itself in the condition to which they have brought it. Moreover, as has been said, we know of no supplementary supportive agent but the trophoblast. Lacking its presence, the initial effort at retrenchment seems to be rapid relinquishment of ground substance.[37] This results in progressive thinning of the mucosa by closer approximation of stromal cells. Even they disgorge something of their cytoplasm, so that eventually this tissue is again of smaller bare nuclei within a reticulum of their short attenuated cytoplasmic strands. A few cells even become pyknotic.

This marked regression at first causes buckling of glands with indentation of their walls. Their lumina are, in turn, squeezed free of stagnant secretion and become narrower.

Of more significance to menstruation is the response of the luxuriated coiled arteries and their superficial capillary network.[27, 28] The previously involved coils and spirals are pressed together and become ever more convoluted. Resistance to blood flow results in diminution of blood volume in the capillaries and indirectly of blood to the stroma depending on it for nourishment. The blood supply is further reduced by tonic contraction of arterial walls, now that progesterone can no longer lessen it. Upon this contraction of arteries is superimposed more frequent and persisting spasmodic constriction of their lumina. This results in such oft-repeated and prolonged ischemia that endothelial walls at various places permit diapedesis into adjacent stroma.

When the lower arterial stream is re-established in the intermittently relaxed vessels, there even occur small hemorrhages into the tissue. As these coalesce in the devitalized upper stromal zones, the former predecidual cells are separated. Some autolyze, releasing a conjectural anticoagulant as well as the postulated menotoxin (necrosin).[40] This substance, drained off by veins, may perhaps act on the adrenal medulla and further increase arterial constriction.

The myometrium, too, no longer subject to the relaxing effect of progesterone, resumes more vigorous contractions of interlacing muscle bundles. These intermittently diminish the stream coming into the basal arteries.

With progressive interstitial hemorrhage and cellular necrosis, somewhat generalized disruption of tissue structures in the outer zone ensues. From pressure of escaping blood much, but not all, of the binding epithelium fragments; walls of superficial venules are torn open; almost all the terminal capillaries, together with the ends of many arterioles and even of some glands, are added to the bloody effluvium in the uterine cavity, now augmented by direct bleeding from the opened vessels. Menstruation has occurred, and will soon be evident to disturb the woman (or comfort her, as the case may be).

Regression

Gradually, as the myometrium recovers more of its uninhibited tone, its criss-cross bundles restrain outward circulation. This, together with the repressive effect of contraction of coiled arteries and the depletion of the anticoagulant coming from stromal autolysis, permits clotting at arteriolar openings and in the rents of veins. The sturdier capillaries of the less superficial mucosal zones are able to maintain their function during the regressive phase. They soon bring the increasing, invigorating estrogen, which comparatively pure hypophyseal FSH again evolves in growing follicles. The cells of the upper tattered stroma respond by closing ranks around the straggling interstitial blood cells.

Most significant to reorganization for care of the next ovum is the estrogen-stimulated proliferation of glandular epithelium. From the torn outer ends, cellular growth extends centripetally, and the mucosa of transected glands joins with the undesquamated segments of the binding epithelium to reline the uterine cavity. Even while catamenia is still going on in some areas of the endometrium, this repair takes place in others, thus covering exposed stroma and the clotted stubs of coiled arteries. Wandering red and white cells and other hematocytes are autolyzed. Menstruation has ceased. Regression has been completed.

Prelude to the Next Cycle

As the glandular epithelium slowly proliferates, it also secretes small amounts of fresh mucus into the lumina. Frequently recurrent contractions of the myometrium, now encouraged by the same estrogen, force this secretion into the central cavity. This estrogen-dominated, postmenstrual, preovulatory yet secretory phase is characterized not only by actual proliferation and functioning of the glands, but also by a gradual increase in the extracellular ground substance of the stroma.

The aggrandizement of material within the interstices of the stromal reticulum produces most of the moderate preovulatory thickening of the endometrium. Mitoses in the stromal cells are comparatively few, but the cells themselves are spread further apart. Thus, as the ovarian follicles stir toward maturity and the complete maturation and final rupture of one, the *post*menstrual, *pre*ovulatory phase of the next cycle progresses.

Summary

From current concepts of embryogenesis and of the sequential hormonally modified enzymology and histology of the endometrium emerges a plausible exposition of menstruation.

Ovarian hormones, periodically varying in quality and quantity, evoke, in the endometrium, growth and function of secretory tubules. Their epithelial rims extend laterally, each joining its neighbor, thus to line the uterine cavity.

Under the influence of estrogen, normally enhanced by progesterone, an unbranched spiral artery in each radiating endometrial segment finally flowers into a tight terminal network of superficial capillaries. These, with their associated venules, are evolved to make maternal blood accessible to an expectant trophoblast.

To defend endothelial integrity in spite of breach by the ovum or dilatation in response to syncytial draining of blood, the reticulated weak matrix surrounding all vessels gradually transmutes (principally by action of progesterone) into a strong tissue of contiguous stromal cells. Such is the endometrium about two weeks after ovulation.

If, however, no trophoblast exists to revive the normally regressing corpus luteum, decrease of estrogen and progesterone deprives this predeciduum of essential nutriment. Stromal cells shrink; autolysis of some attracts leukocytes; the tissue thins. The superficial young capillaries are robbed of firm stromal support and may even also autolyze. The spiral arteries, with caliber lessened by hormonal withdrawal, continue their intermittent contractions. These cause recurrent ischemia, which aggravates the nutritional deprivation of superficial tissue. Spotty endothelial breakdown ensues.

Coalescence of resulting interstitial hemorrhages disrupts the superficial stroma and its dependent vessels, decapitates most glands and many spiral arteries, and disengages much of the surface mucosa. Menstruation begins.

Repair starts promptly as released follicular growth augments estrogen. Endothelium repairs itself. Outward mucosal proliferation of the raw ends

of glands re-establishes the uterine lining. Debris is resorbed from the compacted, still reticulated stroma. Intracavitary menstruation ceases.

For a few days the narrowed arteries remain restful, but soon participate in preparing for a more successful ovum.

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THE NATURAL HISTORY OF MENSTRUAL DISORDERS

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Many patients seen for adult menstrual abnormalities, particularly those for which no etiology can be found, date the onset of their symptoms to menarche. This study attempts to determine the relationship between adolescent menstrual patterns and adult menstrual abnormalities.

Selection of Cases

The eight hundred forty-nine cases reviewed in this study were considered to have a significant variation from a cyclic menstrual pattern. They are a selected group since they were referred to the endocrine clinic from the gynecology clinic of the Sloane Hospital for Women, New York, N.Y. because an endocrine etiology for the menstrual abnormality was suspected. This selection eliminated most patients with definite palpable pelvic pathology as well as those with abnormal bleeding cured by curettage.

An intensive effort has been made to follow the course of all patients seen since 1940. Follow-up is considered inadequate unless the menstrual pattern two years after the initial visit is known. Much information and histological material have been supplied by other hospitals and physicians.

Classification of Menstrual Abnormalities

ETIOLOGY

Etiology Known

Patients placed in the classification of Etiology Known include those with elevated gonadotropins, ovarian tumors, definite thyroid or adrenal abnormalities, tumors involving the pituitary, severe systemic disease, or definite episodes of psychic trauma.

Postpartum. All patients seen for postpartum menstrual abnormalities are grouped together. These represent a group of women known to have been fertile previously, although varied etiologies and sometimes no etiology were found.

Benign intrauterine pathology. In spite of careful screening, benign intrauterine pathology was identified as a cause for frequent noncyclic bleeding in sixty-one cases. These cases are presented only for comparison of their adolescent menstrual pattern.

Etiology Unknown

Patients were placed in the classification of Etiology Unknown if intensive investigation failed to reveal a cause for the abnormal menstrual pattern or if history and physical examination seemed to indicate to the examiner that extensive investigation was not warranted. Many of these patients have had long periods of follow-up, and few originally placed in this category could be changed to another.

Polycystic ovaries. Patients with menstrual abnormalities associated with polycystic ovaries are arbitrarily considered a subgroup of this category, since this diagnosis often cannot be made without visualization of the ovaries and, although the therapy is successful, the etiology remains unknown. This emphatically does not mean to imply that all menstrual abnormalities of unknown etiology are due to polycystic ovaries.

Classification of Menstrual Patterns

The characterization of the type of menstrual abnormality is difficult. There are almost as many patterns as there are patients.

Cases were placed in the primary amenorrhea category if spontaneous menses had not occurred by the age of eighteen.

The designation of amenorrhea was used if spontaneous periods had not occurred for a period of at least twelve months, and oligomenorrhea if menses occurred at intervals of from two to eleven months. Intervals of less than two months were not considered abnormal unless the cycles varied by more than 100 per cent in length.

Patients were placed in the noncyclic bleeding category regardless of the interval between menses if the bleeding, when it occurred, was in any way abnormal. This category therefore includes patients with basic patterns of amenorrhea or oligomenorrhea associated with profuse, prolonged, or intermittent scant bleeding, as well as those with frequent episodes of noncyclic bleeding. The category of moderate was used if the bleeding did not require curettage or hormonal therapy to control it, and severe if these measures were used. This is a biased method of separation since many of these patients were followed with endometrial biopsies, and those with abnormal patterns were more apt to have a curettage or hormonal therapy.

Results

The Significance of Adolescent Menstrual Abnormalities

One hundred sixty-five patients were first seen for menstrual abnormalities by the age of 20 years. The etiology was determined in 19 instances, and 19 resumed normal menstruation at some subsequent time after ovarian wedge resection. These will be considered in more detail later. One hundred and twenty-seven of the adolescents were considered to have no known etiology for their menstrual abnormality. Twenty-four of these were not followed long enough to determine the adult menstrual pattern, and have been classified as not followed. The adult menstrual pattern of the 103 followed adolescents is shown in TABLE 1. Approximately one fourth of the group established relatively normal adult menstrual patterns. An abnormal menstrual pattern continued in 9 of 13 adolescents seen with amenorrhea, in 28 of 32 seen for oligomenorrhea, and in one half of those with moderate noncyclic abnormal bleeding, but in 29 of 34 of those with severe noncyclic bleeding.

TABLE 1

ADULT MENSTRUAL PATTERN OF WOMEN SEEN FOR ADOLESCENT MENSTRUAL ABNORMALITIES OF UNKNOWN ETIOLOGY

Adolescent menstrual pattern	No. cases	Adult menstrual pattern	
		Normal	Cont. abnormal
Amenorrhea	13	4	9
Oligomenorrhea	32	4	28
Noncyclic bleeding			
Moderate	24	12	12
Severe	34	5	29
Total	103	25	78

TABLE 2

SUBSEQUENT PREGNANCIES IN WOMEN SEEN FOR ADOLESCENT MENSTRUAL ABNORMALITIES OF UNKNOWN ETIOLOGY

Adolescent menstrual pattern	Adult menstrual pattern	
	Normal Pregnant/Married	Cont. abnormal Pregnant/Married
Amenorrhea	2/2	1/5
Oligomenorrhea	2/2	10/15
Noncyclic bleeding		
Moderate	4/4	0/3
Severe	0/1	0/11
Total	8/9	11/34

Forty-three of these 103 patients are known to have married. Nearly all with normal adult menstrual patterns are known to be fertile (TABLE 2). Of those with continuing abnormal menstrual patterns, only 1 of the 5 with amenorrhea and none of those with abnormal bleeding have proved fertility. However, two thirds of those with oligomenorrhea are known to be fertile. It would appear that, in a series of patients selected as these were, a pattern of oligomenorrhea developing during the adolescent years is apt to continue into adult life, but that it does not necessarily mean decreased fertility. Adolescents with amenorrhea and severe noncyclic bleeding apparently have a less favorable prognosis.

THE SIGNIFICANCE OF PRIMARY AMENORRHEA

Forty-four patients were seen because of primary amenorrhea (TABLE 3). This diagnosis was considered logical if menstruation had not occurred spontaneously by the age of 18. Twenty-two of these cases appeared to have a congenital cause for their amenorrhea or had elevated gonadotropins. Two had endometrial tuberculosis. One patient with hyperthyroidism started menstruating at the age of 24 years after appropriate therapy and later had 2

TABLE 3
ETIOLOGY OF PRIMARY AMENORRHEA

Etiology	No. cases	Menarche
Primary ovarian failure or congenital abnormality	22	
Endometrial tuberculosis	2	
Hyperthyroidism	1	1 (age 24)
Polycystic ovary		
Resected	3	3 (age 18 to 21)
Not resected	3	
Unknown etiology	13	6 (age 18 to 25)
Total	44	10

TABLE 4
MENSTRUAL HISTORY OF WOMEN SEEN FOR AMENORRHEA, OLIGOMENORRHEA OR ANOVULATORY BLEEDING OF KNOWN ETIOLOGY

	No. cases	Age when first seen	
		Under 20	Over 20
Regular menses established			
Normal to age 20	75	—	75
Abnormal by age 20	34	15	19
Abnormal since menarche	18	4	14
Total	127	19	108

pregnancies. Three patients had the onset of normal menstruation after ovarian wedge resection. Three had a clinical picture compatible with polycystic ovaries, but were not treated surgically. One of these developed scant continuous bleeding at the age of 30 and is included in a later category of patients.

No logical explanation was found for the primary amenorrhea in 13 instances. Six of these started to menstruate at a later age. Five women in this group had pregnancies.

ADOLESCENT MENSTRUAL HISTORY OF WOMEN SEEN FOR AMENORRHEA, OLIGOMENORRHEA, OR ANOVULATORY BLEEDING OF KNOWN ETIOLOGY

A plausible etiological factor for amenorrhea, oligomenorrhea, or anovulatory bleeding was found in 127 women studied (TABLE 4). Nineteen of these were first seen before the age of twenty years. Fifteen of these adolescents had established regular cycles for a period of time before the onset of the menstrual abnormality, and 4 dated the onset of the menstrual abnormality to menarche. Of the total group of 127, 109, or 86 per cent, with menstrual disturbances of known etiology gave a history of normal cyclic menses before the onset of the irregularity.

Eighty-nine patients were seen for the first time because of postpartum men-

TABLE 5

MENSTRUAL HISTORY OF WOMEN SEEN FOR POSTPARTUM MENSTRUAL ABNORMALITIES

	No. cases
Regular menses established	
Normal to age 20	78
Abnormal menarche, normal by age 20	1
Abnormal since menarche	10
Total	89

TABLE 6

MENSTRUAL HISTORY OF WOMEN SEEN FOR ABNORMAL BLEEDING DUE TO BENIGN INTRAUTERINE PATHOLOGY

	No. cases
Regular menses established	
Normal to age 20	57
Abnormal menarche, normal by age 20	2
Abnormal since menarche	2
Total	61

strual abnormalities (TABLE 5). The large majority of these previously fertile women denied abnormal menses during adolescence. One had irregular cycles during adolescence, but was normal by the age of 20, and only 10, or approximately one ninth of the total group, dated the onset of menstrual irregularity to menarche.

Benign intrauterine pathology was found as the cause of a noncyclic bleeding pattern in 61 women (TABLE 6). Four of these women had irregular menses during adolescence, and 2 of these 4 were normal by the age of 20.

These data indicate that women with menstrual abnormalities of known etiology usually have had regular cyclic menses during adolescence.

ADOLESCENT MENSTRUAL HISTORY OF WOMEN SEEN FOR MENSTRUAL ABNORMALITIES OF UNKNOWN ETIOLOGY

Three hundred and nineteen patients with menstrual abnormalities of unknown etiology were first seen beyond the age of 20, and their adolescent menstrual history was reviewed (TABLE 7). It is acknowledged that retrospective menstrual histories lack the desired accuracy. However, the duration of the abnormal menses could often be confirmed through records of previous therapy or investigation.

In marked contrast to women with menstrual abnormalities of known etiology, 248, or 78 per cent, dated the onset of the menstrual abnormality to menarche.

Approximately one third of the patients with amenorrhea of unknown etiology are known to have had a pregnancy after the onset of the amenorrhea

TABLE 7

ADOLESCENT MENSTRUAL HISTORY OF WOMEN SEEN FOR MENSTRUAL ABNORMALITIES OF UNKNOWN ETIOLOGY

Adult menstrual pattern	No. cases	Adolescent menstrual history	
		Normal	Abnormal
Amenorrhea	49	11	38
Oligomenorrhea	154	32	122
Noncyclic bleeding			
Moderate	46	12	34
Severe	70	16	54
Total	319	71	248

TABLE 8

PREGNANCIES OCCURRING AFTER ONSET OF MENSTRUAL ABNORMALITIES OF UNKNOWN ETIOLOGY

Adult menstrual pattern	Adolescent menstrual pattern	
	Normal Pregnant/Married	Abnormal Pregnant/Married
Amenorrhea	3/8	9/27
Oligomenorrhea	3/26	53/95
Noncyclic bleeding		
Moderate	0/4	12/21
Severe	0/13	12/43
Total	6/51	86/186

(TABLE 8). Three of these were over the age of 40 at the time pregnancy first occurred.

Of the 26 women developing oligomenorrhea after a normal adolescent pattern, 3 had pregnancies, while 53 of the 95 patients with oligomenorrhea dating to adolescence became pregnant. The cause for this difference has not been found.

Approximately one half of the patients with moderate noncyclic bleeding have proved their fertility, but only 12 of the 56 patients with severe noncyclic bleeding had pregnancies.

These results suggest that oligomenorrhea present since menarche is frequently a normal variation of the usually accepted pattern, but that severe noncyclic bleeding associated with any menstrual rhythm deserves further consideration. The eventual outcome for this last group will be presented later.

MENSTRUAL PATTERN ASSOCIATED WITH POLYCYSTIC OVARIES

Seventy-two patients selected from the total group of 849 patients had polycystic ovaries identified at the time of laparotomy. Some of these cases were previously reported by Buxton and Vande Wiele (1954). The associated

TABLE 9

MENSTRUAL PATTERNS OF WOMEN WITH POLYCYSTIC OVARIES

Menstrual pattern	Basic pattern	No. cases Plus abnormal bleeding
Primary amenorrhea	3	—
Secondary amenorrhea	18	4
Oligomenorrhea	46	22
Persistent bleeding	5	5
Total	72	31

TABLE 10

ADOLESCENT MENSTRUAL HISTORY OF WOMEN WITH POLYCYSTIC OVARIES

	No. cases
Regular menses established	
Normal to age 20	0
Abnormal by age 20	20
Abnormal since menarche	49
No menarche	3
Total	72

menstrual pattern is shown in TABLE 9. Thirty-one cases, regardless of the basic menstrual pattern, had associated abnormal bleeding patterns.

Twenty women established regular cycles for a short period after menarche, but all were abnormal by age 20. Forty-nine of the 72 dated the onset of the irregularity to menarche, and 3 had primary amenorrhea (TABLE 10).

THE SIGNIFICANCE OF SEVERE NONCYCLIC BLEEDING

A total of 140 patients has been seen for significant noncyclic bleeding. Thirty-one of these had surgically proved polycystic ovaries. The remaining 109 are from the combined series of adolescents and adults with severe noncyclic bleeding of unknown etiology presented previously. Only 16 of the entire 140 patients failed to date the onset of the menstrual irregularity to their adolescent years.

Of the 109 patients originally classified as having severe noncyclic bleeding of unknown etiology, 22 have not been followed for a period of 2 years (TABLE 11). Nine are apparently normal. Sixty-seven of the total group were married. Twelve had proved pregnancies occurring after the onset of their symptoms. Seven women not dating the onset of the abnormality to menarche had pregnancies before the onset of abnormal bleeding.

Seventy-eight followed patients continued to have abnormal bleeding. Thirty-two of these were under 26 years of age when last seen and 46 were beyond this age.

Eleven of the 46 women who were 26 years of age or older have had hysterectomies done because of abnormal bleeding.

TABLE 11
SUMMARY: NONCYCLIC BLEEDING OF UNKNOWN ETIOLOGY

	Age last followed	
	Under 26	Over 26
Inadequate F.U.	18	4
Normal	6	3
Continued abnormal	32	46
Hysterectomy (for carcinoma 4)		11
Under 40		28
Over 40		
Postmenopausal		1
Pregnant (at ages 43, 44)		2
Still abnormal		4
Total	56	53

Only 7 of the remaining 28 patients are over the age of 40. One of these is postmenopausal and symptom-free at the age of 46. Two were pregnant for the first time after life-long histories of abnormal bleeding, and 4 continued to have abnormal bleeding.

Twenty-seven of the entire followed group had either a proliferative or secretory endometrium associated with the abnormal bleeding. Six of these women are apparently normal. One had a hysterectomy at the age of 29 because of severe bleeding. The endometrium at the time of hysterectomy is unknown.

Forty-nine patients had cystic glandular hyperplasia of the endometrium at some time during their study. Six of these are apparently normal. Three had hysterectomies before the age of 38. The endometrium at the time of hysterectomy was secretory in 1, showed cystic glandular hyperplasia in 1, and is unknown in the third case.

Fifteen patients had adenomatous hyperplasia of the endometrium diagnosed at some time during their study. None of these became normal. Seven had hysterectomies. The endometrium at the time of hysterectomy was secretory in 1, aged 40; showed cystic glandular hyperplasia in one, aged thirty; and exhibited adenomatous hyperplasia in a third at age 29.

In 4, a diagnosis of endometrial cancer associated with marked adenomatous hyperplasia was made. These 4 cases are summarized in TABLE 12. The case histories follow.

Case 1. Menarche occurred at the age of 13. Menses were infrequent and associated with profuse and prolonged bleeding. The first curettage was done at the age of 15 and revealed a cystic glandular hyperplasia of the endometrium. This patient was followed in the Sloane Hospital endocrine clinic for 1 year during adolescence, and progesterone was helpful in controlling the bleeding. The history obtained from another hospital reported 7 additional curettages during the ensuing years. At the age of 31 an abdominal exploration was done to rule out an ovarian tumor as the cause of the abnormal bleeding. The operative report described the ovaries as enlarged and polycystic with a thick-

Table 12

History of Women Developing Carcinoma of the Endometrium

Case No.	1	2	3	4
Age at hysterectomy	32	30	30	42
Curettage	8	4	1	12
CGH	Yes	Yes	—	Yes
AH	Yes	Yes	Yes	Yes
Menstrual abnormality				
Abnormal bleeding	Yes	Yes	Yes	Yes
1° Amenorrhea			Yes	
Oligomenorrhea	Yes	Yes		Yes
Age at onset	13	12	1^0	16

ened capsule. The postoperative diagnosis was Stein-Leventhal syndrome. The ovaries were not biopsied. The endometrium at this time was markedly hyperplastic. A curettage 1 year later revealed adenocarcinoma of the endometrium. The patient was treated with radium, and a hysterectomy was done in 1957 at the age of 32.

Case 2. This patient had had abnormal menses since menarche at the age of 12. Her history is very similar to the one above. This case has previously been reported by Gusberg (1947). A hysterectomy was done in 1943 at the age of 30 for carcinoma of the endometrium. The patient was asymptomatic at the time of her last follow-up in 1955.

Case 3. This patient was first seen at the age of 30 with the complaint of infertility and primary amenorrhea. She had had irregular bleeding unrelated to hormonal therapy for a period of 6 months. Previous therapy included pituitary and ovarian irradiation, as well as hormonal therapy.

The ovaries were enlarged and considered to be the etiology of her primary amenorrhea. However, an endometrial biopsy revealed an endometrial adenoacanthoma. She was treated with radium, and a hysterectomy was done in 1957 at the age of 30.

Case 4. This patient had her first curettage at the age of 16. It revealed a cystic glandular hyperplasia of the endometrium. During the ensuing years she had 11 more curettages, and at the age of 42 a hysterectomy was done because of continued abnormal bleeding. An adenoacanthoma of the endometrium was an incidental finding.

Abnormal Bleeding Cured by Wedge Resection of the Ovaries

Thirty-one women with abnormal bleeding were treated by extensive wedge resection or medullectomy of the ovaries and responded to the surgical procedure with normal cyclic ovulatory menses.

The endometrium was of a normal proliferative type in 13 cases. Cystic glandular hyperplasia of the endometrium was present in 9 cases, and adenomatous glandular hyperplasia in the remaining 9 (table 13).

Robinson in 1935 and other authors since have suggested wedge resection of the ovaries as a cure for endometrial hyperplasia. These additional 9 cases

TABLE 13
ENDOMETRIAL PATTERN OF WOMEN WITH NONCYCLIC BLEEDING CURED BY WEDGE RESECTION

Endometrial pattern	Total	Age at operation	
		25 or under	26 or over
Proliferative	13	4	9
Pregnant/married		0/2	2/8
Cystic glandular hyperplasia	9	7	2
Pregnant/married		1/3	0/2
Atypical hyperplasia	9	3	6
Pregnant/married		1/3	4/6

with adenomatous hyperplasia cured by wedge resection confirm the validity of this suggestion.

The adenomatous hyperplasia was of a mild to moderate degree in 8 and severe in 1. Progesterone therapy controlled the symptoms, but did not alter the endometrial pattern.

The majority of the cases did not have hirsutism or obesity. The abnormal appearance of the ovaries was confirmed by culdoscopy in the atypical cases. One of the nine patients with adenomatous hyperplasia originally had a poor response to a rather meager wedge resection. Ovarian medullectomies were done and the response was satisfactory. Another two cases not shown here may be counted as failures. Wedge resection was attempted through a colpoceliotomy incision. Ovarian cortex was obtained, but the surgical incision did not extend into the medulla. Neither of these patients responded in a satisfactory manner, although one patient has occasional ovulatory menses and no longer has profuse bleeding. The ovarian biopsies showed pathology similar to that of the successful group.

Five of these 9 patients had a total of 8 pregnancies. Three pregnancies ended in abortion between the eighteenth and twentieth weeks. In each instance the fetus was apparently normal. Two pregnancies are current and as yet early. One patient had her first pregnancy at the age of 39 after wedge resection at the age of 38. This pregnancy was carried to term, but death of the fetus occurred 2 weeks before delivery. The patient's second pregnancy at the age of 41 ended in an early spontaneous abortion. In the entire series 1 normal living infant has been obtained so far.

The longest period of follow-up on this group is 3½ years. The youngest patient is now 25 years of age and the oldest is 41. One patient is known to have recurrent atypical hyperplasia following a midtrimester abortion.

In reconsidering the group of women classified as having noncyclic bleeding of unknown etiology, it seems that all patients treated for carcinoma of the endometrium might have responded, at some time in their past, to a less radical procedure such as wedge resection. Eight of the 15 patients with adenomatous hyperplasia still have their uteri. All are under the age of 30. The presence of polycystic ovaries has been confirmed in 2 by ovarian biopsy and in 1 by culdoscopy. Pelvic examination suggests this diagnosis in a fourth.

Although wedge resection leaves much to be desired as measured in terms c successful pregnancy, it is suggested for consideration as an alternative t hysterectomy in the treatment of persistent adenomatous hyperplasia occurrin in the younger age groups.

Summary

The majority of women who developed menstrual abnormalities for which a etiological factor was found had established regular cyclic menses during adolescence. In direct contrast to these, the women with menstrual abnormalitie of unknown etiology or with polycystic ovaries dated the onset of the menstrua abnormality to the adolescent years.

Three-fourths of the adolescents seen for menstrual abnormalities not associated with an identifiable etiology continued to have the abnormal menstrua pattern as adults.

The menstrual pattern of severe noncyclic bleeding was usually establishe during adolescence. The reproductive performance in this group was poo and, of 46 patients followed to the age of 26 or more, 11 had hysterectomie because of abnormal bleeding. Four of these were for endometrial cancer.

The menstrual abnormality of the 4 patients developing endometrial cance dated to menarche, and repeated curettages showed progressive endometria hyperplasia.

Nine patients with adenomatous hyperplasia developed normal ovulatory menses after extensive wedge resection of the ovaries. This method of treatment is suggested as an alternative to hysterectomy in the control of abnorma bleeding associated with adenomatous hyperplasia of the endometrium i younger women.

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Discussion

SOMERS H. STURGIS (*Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass.*): From her title, I had no idea how Anna L. Southam might develop her thesis. She might have taken a phylogenetic view; this would have meant, perhaps, tracing the earliest origin of the formation of estrogen as the hormone necessary for maintaining in good function the oviduct apparatus in lower forms of life so that ova might be extruded efficiently to the exterior and there take their chances of union with a spermatazoon. Then, she would have noted the evolution of internal fertilization and, finally, of placentation, and the development of a corpus luteum and then a placenta to meet these demands.

On the other hand, she might have followed the steps in ontogeny: first, the neuter phase of prepuberty in the young girl; then the first appearance of estrogenic effects, breasts and physical development; then the menarche, and estrogen withdrawal type of flow for the most part; thereafter, the achievement of ovulation and a mature reproductive cycle; and, finally, a primary ovulation failure in the fifth decade before eventual ovarian deficiency and diminution of estrogenic substances. In fact, Southam has chosen this approach but has limited it to the span of life from adolescence to maturity in patients seen between 1940 and 1955. Either approach lends itself to the conclusion that successful ovulation and corpus luteum function is the supreme manifestation of the most mature and complicated physiological interplay of the endocrines involved in the nonpregnant menstrual cycle.

I am convinced that this is true. If ovulation recurs with regularity, we can in most cases exonerate the pituitary-ovarian axis of primary responsibility for menstrual irregularities. The interlocking mechanisms that must function correctly if rupture of the ovarian follicle is to occur are the last to be developed satisfactorily as adolescence progresses and the first to be interrupted in the decade before the menopause. It must be stressed that, if it can be demonstrated that ovulation is taking place, then most menstrual disorders must be related to systemic or neoplastic, rather than to endocrine, causes. When ovulation is not accomplished in a mature woman, however, many of the common menstrual disorders take place.

Southam has classified menstrual disorders according to etiology, but she mentions those in whom the etiology has been determined only as part of the group of 849 cases; the bulk of her study concerns those with "etiology unknown," thus considered to be functional disorders. It is difficult to characterize types of menstrual patterns, as she admits. One can say only that an abnormal menstrual pattern is defined as one in which there is either too little or too much bleeding. To this one might add another qualification—bleeding that is quite irregular and unpredicted.

Another type of classification might deal with those who were known to have ovulated at one time or another and those who have never ovulated. I do not find in Southam's paper a discussion of the methods, other than the endometrial biopsy, she used to detect ovulation which, on some rare occasions, she notes as having been secretory.

Her careful study represents a tremendous amount of work. It is packed full of essential and basic information concerning follow-up of different types of cases over several years. It is a presentation so full of extremely important facts, statistics, and tables that it is difficult to digest without careful reading. Indeed, there is so much material here that one could wish that Southam would give us a breakdown with more detailed consideration of many of the fascinating and significant tables. For instance, we find that there were 44 patients who were seen because they had had no periods up to the age of 18. Twenty-two of these cases are said to have had a congenital cause for their amenorrhea or to have had elevated gonadotropins. This is an important group, and we should like to know what the congenital abnormalities were. If 50 per cent of the girls who reached the age of 18 without a period have a congenital malformation or dysfunction of the ovaries, this is certainly a most significant percentage. It would be of tremendous interest also to know what happened to these 44 patients, particularly the 22 who did not have any congenital cause for their amenorrhea as they developed into maturity. I cannot find this group mentioned again in any later report. In Southam's series, 72 patients had polycystic ovaries demonstrated at laparotomy. This is a very important group of patients. Almost one half of these had abnormal bleeding patterns, and the same number apparently responded to wedge resection with normal cyclic ovulatory menses thereafter. A study of these 72 polycystic ovary cases would in itself be a most rewarding investigation. For what reasons did more than 50 per cent fail to respond to wedge resection? One might hope that a perusal of these 72 cases would give us some clue to the time at which this operation was indicated. It is of interest to note that 9 who did respond to the operation were found to have had cystic glandular hyperplasia of the endometrium. A small but interesting point is the mention of 3 cases that had less than adequate wedge resection with a failure perhaps due to the fact that the incision did not enter the ovarian medulla. Southam notes that progesterone controlled but did not cure the hyperplasia associated with abnormal bleeding in some of these polycystic ovary cases. She suggests finally that cystic or adenomatous hyperplasia often may be associated with a polycystic ovarian development and, if persistent, that it may then lead to carcinoma of the endometrium, as was found in 3 of her present cases. This leads to her suggestion that a wedge resection prior to development of cancer certainly would have been a preferable procedure.

It is to be hoped that Southam, having done this extensive cursory survey of more than 800 cases, will have the opportunity to break down the various groups of cases and give us details that will lend themselves to some interpretation of causes for the disorders. One may conclude that cases of amenorrhea or severe noncyclic bleeding through adolescence have a relatively poor prognosis of fertility when they marry, and that these younsters, before they reach their twenties, should have much more attention in an attempt to remedy their condition. Finally, we must recognize the extent to which a statistical survey falls short of the ultimate goal of revealing the causes of these disorders. Perhaps it is necessary to study single cases in great detail. Certainly, we cannot as yet guess wherein lies the endocrine failure. In some it may certainly be the pituitary; in others the ovary itself obviously is at fault; in still others perhaps the endometrium is nonsensitive to the proper ovarian stimulation. When and if it may become possible to break down the causes for menstrual abnormalities according to the physiological etiology, then we may hope to have a more effective means of treating these distressing problems.

Regarding the theories of menstruation, in 1946, George Corner[1] wrote: "Menstruation is a paradox and a puzzle—a normal function that displays itself by destruction—a phenomenon seemingly useless and ever retrogressive, that exists only in higher animals—an unexplained turmoil in the otherwise serenely co-ordinated process of uterine function." One must admit even now that there are episodes in this process that are puzzling and unexplained.

The paradox of menstruation refers to the unique distinction of the endometrium as a tissue that, to be normal, must undergo recurrent slough and necrosis. Nowhere else in the body can such pathological changes be considered the criteria of health rather than disease; nowhere else in the adult do we find such an unusual exhibition of growth and repair, except in response to external injury or internal disease.

An understanding of the mechanism of menstruation must necessarily proceed from gross and microscopic observations—and theories that may link these observed changes together sequentially can be strengthened by our knowledge of the purpose served by the endometrium in the economy of racial survival.

As a working hypothesis, we can do no better than accept Corner's interpretation of the endometrial cycle. In this view, the development of the unique spiral arteries, the progravid changes, and even menstruation itself are all an expression of the provision of maternal blood to the ovum until the placental circulation is established. In the latter connection, Corner points out that in rhesus monkeys there is always some extravasation of blood at the implantation site that reaches the exterior, and similar extravasations in the vicinity of the ovum were found in two of Hertig and Rock's human series[2], as well as in the early implantations described by Brewer.[3]

There are other morphologic changes secondary to this extravasation. The collection of edema fluid superficially in the progravid phase has been mentioned. Incidentally, I think we should accept this term, as suggested by Bartelmez, as being better than "secretory phase" for the postovulatory half of the cycle, since this same investigator has pointed out that glandular secretion does occur in the follicular phase as well. Recently Bartelmez has explained this collection of edema fluid as a result of the continued growth of the spiral arteries, which causes increase in their coiling and kinking and a resultant decrease in blood flow. The edema fluid itself spreads out the capillary net, and it may facilitate implantation and trophoblastic invasion with a lesser risk of that degree of hemorrhage that might otherwise wash away the tiny conceptus.

Certainly the intermittent contractions of the spiral arterioles still constitute one of the puzzles. There is no doubt that some mechanism accounts for the spasmodic spurts followed by shutoffs that Markee observed in transplants in the monkey's eye. Something is responsible for alternate constrictions and relaxations of the spiral arteries; otherwise, inordinate and exsanguinating hemorrhage would inevitably accompany the first menstrual flow. The idea that a vasoconstrictor enzyme might be released by degenerating endometrium has had little corroboration. Such an enzyme would necessarily be released prior to, or at least concomitantly with, initial sloughing. In his recent superb review of serial endometrial sections, Bartelmez[4] shows that a luminal constriction of the radial arteries in the myometrium occurs frequently through the cycle. More important, he has measured the lumen and the over-all diameter of regions of constriction, and it appears from his observations that the radial arteries in the myometrium do not contract, but become locally elongated; this stretching causes the reduction in lumen and blood flow that is mainly responsible for ischemia of the surface endometrium. This is not due to forceful myometrial contractions, since the myometrium is flabby; its atonicity is partly responsible for the accumulation of viscous secretions in endometrial glands. We do not know what causes this apparent local and intermittent arterial stretching, although the evidence now appears to indicate this as the reason for decreased blood flow, rather than contractions of the vessel walls or constriction from myometrial pressure.

We must acknowledge the role of the predecidua cells developing as a supportive buttress about the small capillaries, must consider the possibility that in the case of failure of implantation and the resultant corpus luteum decline these cells first feel the effects of lessened blood flow. When they break down from malnutrition, the capillary bed rapidly becomes vulnerable to autolysis and breakdown. This forces us back once more to acceptance of hormone deprivation as the cause for decreased blood flow due to the local areas of stretching—not contraction—of the radial and spiral arterioles, as demonstrated by Bartelmez. The "why" and "how" of this mechanism still elude us.

An observation by Hisaw[5] suggests that we may still find the answer in enzymatic systems sensitive to circulating steroids. He observed that if one dropped an infinitesimal amount of crystalline progesterone, as little as gamma, on the surface of a marsupialized follicular phase endometrium in rhesus, breakdown and bleeding first appeared focally, and then spread rapidly to involve the whole uterine lining. This could best be explained, he believed, by some catalytic action that triggered an enzyme system responsible for the more widely spread endometrial involution.

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Part VIII. Structural and Functional Aspects of the Placenta

COMPARATIVE ANATOMY OF THE PLACENTA

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The wide scope of the subject matter with which this paper is concerned precludes the possibility of discussing fully all its various important aspects. Sundry features of placental morphology have been fully discussed in recent reviews (Amoroso, 1952, 1955a, 1955b, 1955c; Bøe, 1953, 1954; Ramsey, 1955; Boyd, 1955; Matthews, 1955). Consequently, an extensive account of placental histology, constituting as it would much that is repetitious, is not contemplated. On the contrary, the observations to be presented are rather miscellaneous in character and are intended to supplement and extend, and also to clarify to some extent, the knowledge derived from earlier investigations.

The Evolution of Viviparity

During the transition from oviparity to viviparity in fishes, reptiles, and mammals, profound changes have occurred in the nature of the egg and in the structure of the female animal. These evolutionary innovations have involved: (1) a decrease in the number of eggs shed; (2) perfection of internal fertilization; (3) utilization of the yolk for absorbing secretions and other nutrients as well as for the elimination of waste products; (4) intrauterine care of a few young to relatively advanced stages in development; (5) participation of endocrine structures in extending gestation, with the object of retaining eggs in the genital tract for periods beyond the limits of the estrous cycle; and (6) adaptation of the endocrine control of lactation to preparation for early nutrition of the newborn.

The most primitive type of development is that in which innumerable sperm and innumerable minute, naked eggs poor in yolk were shed into the surrounding water, where fertilization was effected without the participation of the parents themselves. With the adoption of a terrestrial habitat, however, certain new requirements to ensure survival became necessary. The first of these was for some association of the sexes, which may have begun as the result of chemical stimulation due to substances extruded at spawning time. Another was for some sort of protection against desiccation. In addition to the secretion of a protective envelope around the egg, developments for assuring greater survival have included increased storage of nutritive material in the form of yolk and a concomitant reduction in the number of eggs produced. Moreover, the production of a large cleidoic egg would, in general, presuppose a parallel development of sexual association to ensure internal fertilization before the egg was completely closed off from its environment.

With the development of these innovations, an early free-living aquatic existence had now become impossible, and a more protracted developmental period was possible only if there were sufficient reserve food materials, and if the larval stages could be compressed into the embryonic period. The larger and less numerous the eggs became, however, the more valuable they were to the species and, hence, we see arising various kinds of arrangements for the care of the developing embryos. Such close contacts led ultimately to the complete retention of the egg within the parent organism, at some level in the gastrointestinal or reproductive tract, or in some specially prepared marsupium or pouch. Such embryos were provided with ample supplies of yolk and obtained little, if any, additional food from the uterus. This must have been the primitive ovoviviparous state, when the incubation of the embryo in its egg within the uterus was simply for better protection and greater constancy in environment. From this point onward, evolutionary changes in reproduction have involved a loss of yolk concomitant with specialization of the fetal membranes and endometrium as an organ of nutrition and internal secretion. In some such way viviparity arose.

Proceeding and associated with these evolutionary changes was the development of corpora lutea. In vertebrates, these luteal structures are not invariably associated with viviparity. Indeed, they have been described in oviparous and ovoviparous selachians, teleosts, and reptiles (Giacomini, 1891; Cunningham and Smart, 1934; Hisaw and Abramowitz 1937; Rahn, 1938, 1939; Clausen, 1940; Cieslak, 1945; Bretschneider and de Wit, 1947). In mammals, where they achieve their greatest specialization, they first appear in the oviparous monotremes. They are associated in vivipara with the reiention of the embryos in the uterus and in ovipara with the storage of eggs in the oviduct, although a possible relationship with lactation has been suggested tn the oviparous mammals, where also their secretions play an important part in controlling the pattern of the nursing behavior.

In oviparous and ovoviviparous teleosts, the corpus luteum has been shown to produce a secretion, "oviductin" (Bretschneider and de Wit, 1947), which is responsible for the growth of the ovipositor in the bitterling (*Rhodeus amarus*), thus providing the first direct evidence that the secretory function of the corpus luteum is not an evolutionary innovation of the mammalia. Progesterone is not, however, the effective secretion of these luteal structures, nor is it desoxycorticosterone, although both are outstandingly efficient in inducing the rapid elongation of the ovipositor in this species. As with other steroid hormones, the effects of progesterone and desoxycorticosterone are mediated by the anterior pituitary; the action of the natural secretion of the corpus luteum of *Rhodeus* is direct. A case also can be made for the presence of progesterone and progesteronelike agents in the ovaries (Valle and Valle, 1943) and the circulating plasma (Bragdon *et al.*, 1954), respectively, of ovoviviparous snakes. It may not be too much to describe the action of these agents as progestational. Consequently, the possession of a progestin cannot be regarded as an evolutionary innovation in the control of mammalian pregnancy. Evolution in the field with which we are concerned consists of changes of hormone emphasis rather than of kind. It is abundantly evident, therefore, that the origin of the placenta is bound up with the appearance of viviparity in the animal world and hardly can be studied in mammals. It must have been preceded by a great variety of adaptations such as those we find today among the Dipnoi, where Kerr (1900) showed that, in *Lepidosteus*, the male develops papillae on the pelvic limbs during the breeding season. According to Cunningham (1929) these filamentous appendages secrete oxygen for the benefit of the eggs around which they are wrapped and, without doubt, the swamp water in which these animals live is extremely poor in oxygen. For the simplest kind of placenta, however, recourse must be had to selachian fishes, which invented true viviparity, doubtless for purely protective reasons (Amoroso, 1952, 1955a, 1955c), presumably before the reptiles went ashore in the Silurian or Devonian Paleozoic.

Selachian Ovoviviparity and Viviparity

The first step in placental viviparity consists in ovoviviparity. The distinction is based on the difference between live-bearing forms, which retain an egg with enough yolk to carry the embryo through its development, and those in which there is an intimate nutritive connection between mother and offspring; the former are said to be ovoviviparous. Nevertheless, Ranzi's (1934) investigations of selachian ovoviviparity have shown that there are considerable variations in the composition of the uterine fluids that bathe the eggs while the embryo develops. This indicates that, in these ovoviviparous species, the uterine wall, by its glandular activity, already plays an important part in providing nutritive material for the young individual. This is well brought out in TABLE 1. Also it should be observed that, in general, as the weight of the egg at the beginning of development decreases, there is a noticeable increase in the percentage of organic substances in the uterine fluids, which is accomplished with a good deal of alteration in the uterine lining.

In viviparous forms, on the other hand, the development of circulatory structures for transport of metabolic products between mother and embryo is necessary to supplement a reduced yolk supply in the egg. Accordingly, before the advent of the chorioallantoic circulation, the utilization of the yolk sac for absorbing secretions and other nutrients in the uterine fluids, as well as for the elimination of waste products, seemed to be one of the initial adaptations that enabled the fetus to obtain food more directly from the maternal circulation. In a viviparous form such as *Mustelus laevis*, respiratory and ex-

Table 1

Dependence of Eggs on the Uterine Environment in Ovoviviparous Selachians

Species	Weight of egg at beginning of development (gm.)	Weight of egg at end of development (gm.)	Change (per cent)	Per cent organic substance in uterine fluid
Scymnus lichia	130.0	188.0	45	1.6
Acanthias vulgaris	23.0	41.0	78	2.4
Galeus canis	46.9	102.2	118	4.9
Mustelus antarticus	4.4	32.2	635	7.1
Mustelus vulgaris	3.9	60.6	1450	5.1
Trigon violacea	2.0	118.0	5800	13.3

cretory functions presumably are supplied by the development of a primitive type of yolk-sac placenta. In the placental region the yolk-sac ectoderm becomes syncytial and is invaded by capillaries from the mesoderm; the epithelium of the oviduct is reduced in thickness so that, in places, the maternal capillaries are brought into contact with the egg membrane. The fetal and maternal circulations are thus brought into close apposition, separated only by the egg membrane and the respective endothelia. It seems doubtful, in view of the size of the yolk sac, which is as large as that in the oviparous forms whether this simple type of yolk-sac placenta serves any major trophic function although it has been suggested that absorption of organic material, and particularly fats, may take place by this route (Amoroso, 1952, 1955a, 1955c).

The Choriovitelline and Chorioallantoic Placenta of Reptiles

The possibility of an advance in the evolution of viviparity comes with the emancipation from the aquatic environment achieved by the Amniota, and the production of large yolky eggs that undergo their development on land instead of water. Its establishment through the intermediate stages of ovoviviparity is well illustrated by the reptiles, in which stages of perfection of the choriovitelline and chorioallantoic placenta can be traced (Giacomini, 1891; Flynn, 1923; Weekes, 1930, 1935; Cate-Hodemaker, 1933; Boyd, 1942).

In the cleidoic egg of reptiles, the impossibility of eliminating nitrogenous waste products of metabolism by diffusion into the surrounding medium is compensated for by the development of an allantois in which these substances can be accumulated and discharged after hatching. In addition, this new structure assumes a respiratory function and, becoming highly vascularized, is applied closely to the porous shell to become of increasing importance as an organ of gaseous exchange. Whereas egg laying is the more common in some snakes and lizards the eggs are retained in the oviduct until the young are ready to be born. Thus, by retaining the eggs in the oviduct during their development and making use of the vascular allantois in addition to the yolk sac in the production of a placenta, the stage is set for the Amniota to start anew the process of evolving a placenta. The development of these circulatory structures, in time, would supplant the need for yolk and, as the egg became depleted, the yolk sac would be retained as the first vascular structure to meet the needs of the developing embryo. In this way the choriovitelline placenta was formed and, as a highly vascular structure, functioned efficiently until it was superseded by the chorioallantoic placenta.

The Mammalian Yolk-Sac Placenta

A similar modification of the yolk sac, comparable to that encountered in reptiles, is found in marsupial mammals, notably in the opossum (*Didelphys*) and the native cat (*Dasyurus*), where it may function as the only placental connection between parent and offspring (Hill, 1900a, 1900b; Amoroso, 1952, 1955a). Among the Monodelphia, the yolk sac has undergone its highest specilization in the Rodentia, Lagomorpha, Insectivora, and Chiroptera and, when vascularized, the choriovitelline placenta may function as an organ of exchange whose importance is not secondary to that of the chorioallantoic placenta (Everett, 1935; Brambell *et al.*, 1951, Brambell, 1954; Brambell *et al.*, 1948; Amoroso, 1952, 1955a).

By the time the mammalian blastocyst has entered the uterus it has acquired through the mechanism of cleavage the segregation of the embryonal area from the extraembryonic segment. These two parts differ in structure and prospective fate. The extraembryonic segment, which adapts the embryo to the environment of the uterus and provides for its protection and nutrition, comprises the entire trophoblast and that portion of the completed thin-walled yolk sac related to the extraembryonic region of the trophoblast. The embryonic area consists of the embryonic disc and the adjacent portion of the endoderm from which the gut will arise.

Although marked changes in its gross morphology occur, the yolk sac continues to increase in size and histological complexity as gestation advances. At some areas, in association with the trophoblast, it enters into placental relationships with the maternal tissues and, throughout development, may play an important physiological role. Two major periods, each characterized by a succession of phases, may be recognized in the developmental history of the yolk-sac placenta in Rodentia, Lagomorpha, Insectivora, and Chiroptera. In the first period, the omphalopleure or fetal constituent is nonvascular, and passes through two phases, being first bilaminar and later trilaminar. In the second period the omphalopleure is vascularized by fetal vessels. In the first phase the embryonic segment of the omphalopleure is vascularized to form the choriovitelline placenta; in the second phase the choriovitelline placenta disappears as the exocoelom diverts the blood supply by separating it into vascular splanchnopleure and nonvascular somatopleure. This separation takes place with varying rapidity and completeness, depending on the species. In some, such as the ungulates and carnivores, the extension of the extraembryonic coelom leads to the establishment of a complete chorion and a yolk-sac vesicle with an independent wall of its own. In others, the coelom does not spread so far, and the vitelline vessels continue to advance into the extraembryonic segment of the yolk sac and, sporadically in late pregnancy, a few capillaries may extend beyond the limits of the exocoelom to vascularize the persistent trilaminar omphalopleure. The vascularization of the persistent trilaminar segment of the yolk sac by allantoic vessels from the adjoining somatopleure, on the other hand, is a consistent feature of yolk-sac development.

The Chorionic Placenta of Mammals

The choriovitelline placenta is present as a temporary structure that ceases to exist whenever the exocoelom extends into the area vasculosa and splits it into vascular splanchnopleure and nonvascular somatopleure or true chorion. This portion of the true chorion is apposed to or fused with the endometrial mucosa to form a transient supplementary, nonvascular chorionic placenta. Its splanchnopleuric derivative, on the other hand, is added to the invaginated roof of the yolk sac, forming with the abembryonic omphalopleure the so-called inverted yolk-sac placenta.

The chorionic placenta comes into existence independently of the choriovitelline placenta and obviously represents a step in the formation of the chorioallantoic placenta, since a part of it may later become vascularized by allantoic vessels. This area represents, therefore, a persistent region of chorionic placentation and may, according to Mossman and Weisfeldt (1939) and Amoroso (1952), function in the exchange of materials between the exocoeloma and the uterine lumen.

Histotrophic Nutrition of the Fetus

In the absence of any considerable amount of yolk, the production of histotroph from the uterine secretions is of great importance in many species before the establishment of a close apposition of maternal and fetal vascular structures. Secretion of the uterine glands and surface epithelium occurs quite generally in pregnancy and probably contributes, at various times and in various ways during gestation, toward nourishing the blastocyst. In all mammals, secretion probably contributes to the nourishment of the free blastocyst and, in variable degrees, to the support of the implanting blastocyst. In the Artiodactyla and the Perissodactyla it seems likely that secretion provides a principal source of nourishment for the embryo from the beginning to the end of pregnancy. In the carnivora, the extravasated, disintegrating, maternal blood in the placental or paraplacental hematomas, in so far as it bathes viable and functional parts of the fetal membranes, is absorbed by the columnar cells of the chorionic membrane, thus providing the growing fetus with a source of iron necessary for the production of its own red blood cells. In the other mammals, secretion plays a more subsidiary and variable role, although in the placenta of some rodents there may be a direct communication between the glandular secretion in the subplacental decidua and the intervillous space.

Uterine Milk as a Source of Gonadotrophin

In ungulates, the uterine milk in the lumen of the uterus has long been regarded as a nutritive fluid known to contain protein substances, lipoid droplets, leukocytes, iron compounds, pigments, red blood cells to some extent, and occasional crystals (Bonnet, 1882; Kolster, 1906; Amoroso, 1952). Recently, however, a potent gonadotrophin has been demonstrated in pregnant mares in the tissues comprising the endometrial cups and in the uterine milk emanating from them (Cole and Goss, 1943; Rowlands, 1947; Amoroso, 1952, 1955b; Clegg *et al.*, 1954). The part it plays in gestation is not clear, but it may be concerned with the development and growth of numerous accessory corpora lutea and, essentially, with the maintenance of pregnancy at a critical stage (Amoroso *et al.*, 1948).

Histologically, the cups have a distinctive appearance comparable to the decidual responses of the endometrium during pregnancy in other forms (FIGURE 1). An irregular space in the small area above the uterine depression (representing all that remains of the uterine lumen at the level of the cup) separates the nonvillous chorion from the endometrium. In life, this space contains a brownish gluelike coagulum that is the richest source of pregnant-

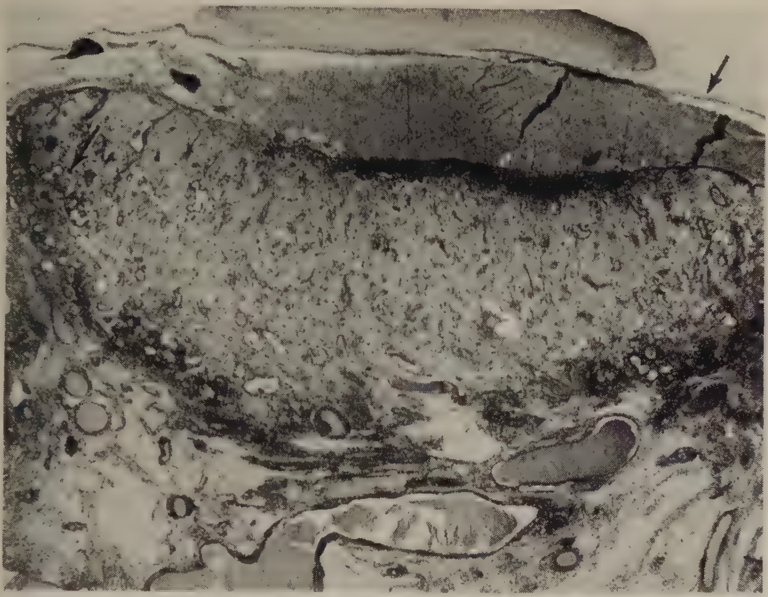

FIGURE 1. Photomicrograph of a section of an endometrial cup of a mare 63 days pregnant When first formed, the cups have flat surfaces and are sharply delimited from the myometrium by condensations of lymphoid tissue and the basal segments of the uterine glands. The glue-like coagulum that has accumulated beneath the nonvillous chorion (arrow) overlying the cup is derived in part from cytolyzing endometrial cup cells and in fat from the glandular secretions. ×8.

mare serum gonadotrophin. Embedded in it are great numbers of degenerating uterine epithelial and decidual cells. The ducts of the glands that open on the margins of this space frequently are filled with a nongranular coagulum containing an abundance of cell debris. By the sixth month of a pregnancy (which lasts eleven months), the quantity of solid precipitated material from the cups is reduced greatly, and during the second half of pregnancy it disappears almost completely.

At the margin of this space, fetal and maternal tissues are intimately apposed. In the central area, on the other hand, the endometrium has lost, not only its surface epithelium, but much of the decidua as well (FIGURE 2). Even the epithelium of the innermost parts of the glands situated at the base of the cup show varying degrees of degeneration. The earliest sign of degeneration is the appearance of two or three perinuclear vacuoles around the nuclei of the decidual cells. Studies now in progress indicate that most of these vacuoles contain PAS-positive material which is saliva resistant. In more advanced stages of degeneration, the small vacuoles enlarge and coalesce to form a single large vacuole that is often situated in one half of the cell, displacing the nucleus to the other. In the stage immediately preceding complete degeneration, the vacuole occupies the entire cell. Finally, the disappearance of the cell membrane permits the escape of the degenerate nucleus and the liquefied contents

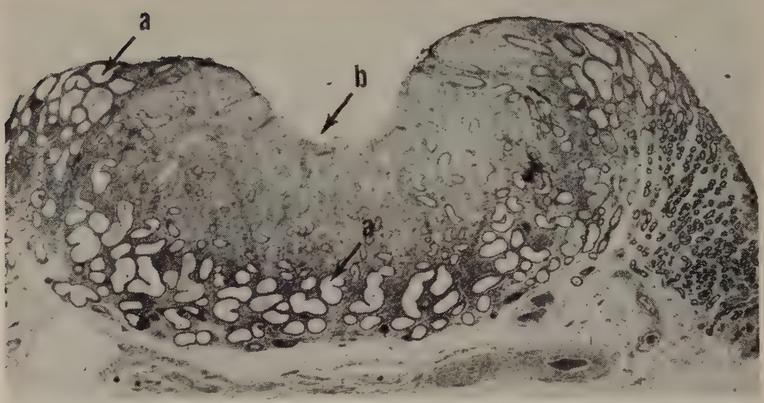

FIGURE 2. Photomicrograph of a fully developed cup from the surface of which the detritic coagulum has been removed. The lips of the depression result, in part, from overgrowth of the margin of the transformed area and partly from the degeneration and desquamation of the central region of the cup (b). Note the dilated fundic parts of the uterine glands (a). ×8.

into the space between trophoblast and endometrium. The uterine mucosa thus provides further histological evidence that it is engaged in physiological activity of importance.

Endocrine Activity of the Placenta During Pregnancy

One of the problems confronting mammals in the evolution of viviparity was that of extending gestation with the object of retaining eggs in the genital tract for periods beyond the duration of the normal estrous cycle. In the duck-billed platypus (*Ornithorhynchus*) and the spiny anteater (*Echidna*), which still lay eggs in true reptilian fashion, the situation is similar to that of other oviparous verebrates, in which internal fertilization occurs and merely involves the retention of the eggs until the shell is formed. Likewise, in marsupials in which pregnancy lasts from 7 to 28 days and in which the luteal phase of the cycle is so prolonged as to be comparable with true pregnancy in duration (complete pseudopregnancy), parturition is correlated with the involution of the corpora lutea. Since in this species the ovaries are necessary throughout gestation (Hartman, 1925), it is assumed that the maintenance of pregnancy does not involve hormonal mechanisms other than those that participate in the regulation of the estrous cycle. In the Monodelphia, on the other hand, as gestation is prolonged, other and more complex controlling mechanisms appear. Evidence implicates the placenta in these changes, the direction of which is toward the endocrinological specialization of this organ as an adjunct to the ovary and the pituitary (Hisaw and Astwood, 1942; Amoroso, 1955b, 1955c).

That an extraovarian source of estrogen and progesterone exists is proved for the human female, the monkey, and the mare by the appearance in quantity of their derivatives in the urine of pregnancy after double oöphorectomy (Allan and Dodds, 1935; Dorfman and van Wagenen, 1941; Hart and Cole, 1934; Amoroso, 1957). That this source is the placenta remains the best hypothesis. In addition, progesterone has been isolated from human and mare placentae (Zander, 1954; Pearlman and Cerceo, 1952; Salhanick *et al.*, 1952; Short, 1956; Amoroso and Laing, 1958; Zander and von Münstermann, 1956), and more recently from the placenta of the Indian rhinoceros (Amoroso, 1957). Likewise, placental gonadotrophins have been shown to be elaborated during pregnancy in the mare (Cole and Saunders, 1935), in humans (Collip, 1930; Browne and Venning, 1936; Tenny and Parker, 1939; Evans *et al.*, 1937), in the monkey (Hamlett, 1937; Hartman, 1939), the chimpanzee (Zuckerman, 1935; Schultz and Snyder, 1935), and the rat (Astwood and Greep, 1938; Ray *et al.*, 1955), and more exactly from the Langhans' cells of the fetal chorion (Wislocki and Bennett, 1943; Stewart *et al.*, 1948; Stewart, 1951). In the mare, the chief source of pregnant-mare serum gonadotrophin (PMSG) is the endometrial cups, which are found on the maternal part of the placenta (Cole and Goss, 1943; Amoroso, 1955b).

Grosser's Classification of the Chorioallantoic Placenta

Biologists, like philosophers, often swing to extremes. In the effort to establish an idea, it is often overstated. For nearly half a century it has been the fashion among physiologists to derive the efficiency of the placenta directly from Grosser's (1909) hypothesis of four morphologic types of chorioallantoic placenta. Grosser's theory of diminishing thickness of layers seemed sound during the period when it was considered possible to explain all placental exchange on the basis of diffusion and filtration. However, placental transfer depends as much, if not more, upon the cytological and cytochemical structure of the placenta as well as the anatomical disposition of the blood vessels supplying the placental membrane, aspects which Grosser's formulation does not take into account.

One of the most ambitious tests of this theory is recorded in a series of papers by Flexner and his associates (Gellhorn *et al.*, 1941; Flexner and Gellhorn, 1942). By the use of radioactive sodium, these investigators measured the transfer of sodium from mother to fetus in species having all the various types of placenta indicated by Grosser. They discovered the remarkable fact that the amount thus transferred across a unit weight of placenta in a unit of time at what they believed to be a comparable stage of late pregnancy varies according to the structure of the placenta. In those with all six layers, the rate of transfer is much less than in those with fewer layers. On this was based the assumption that the fewer the layers the greater the speed of diffusion. This we now know to be a fallacious assumption.

Nevertheless, there have been certain anomalies, obvious and unanswered at that time, for which answers are still far from clear. The most notable of these is the passage of water. We still do not know, for certain, the forces concerned in the passage of water into the developing ovum at all stages of its growth. In regard to other materials, it is sufficient to point out that, for lipids (Popják, 1954), for steroids (Parkes, 1954; Jost, 1954), and for immunological quantities of protein (Brambell, 1954), the evidence indicates that we are dealing with active cellular transport by the placenta. Widdas (1952) and Huggett (1954) showed that the passage of glucose across the placenta of the sheep could not be explained by a diffusion mechanism, but needed some type of carrier system in the cells of the placenta. We have no evidence as to the nature of these carriers, although their possible identification with the hexose esters of the Embden-Meyerhof cycle has been postulated (Huggett, 1954).

The Maternal Placental Circulation

An important aspect of pregnancy pertains to the anatomical arrangement and the physiological control of the maternal blood vessels supplying the placenta. Of interest in this connection is the character of the placental vessels in the hyena (*Crocuta crocuta*). In vertical sections of the placental zone similar to and more advanced than those represented in FIGURES 3, 5, 7, and 9, the following zones are recognizable: (1) the chorionic plate, (2) the trophoblastic zone, (3) the attachment zone, (4) the deep glandular zone, and (5) the marginal zone.

The chorionic plate. This zone comprises a layer of syncytial trophoblast contiguous to the mesoderm of the chorionic plate and provides the lining of an

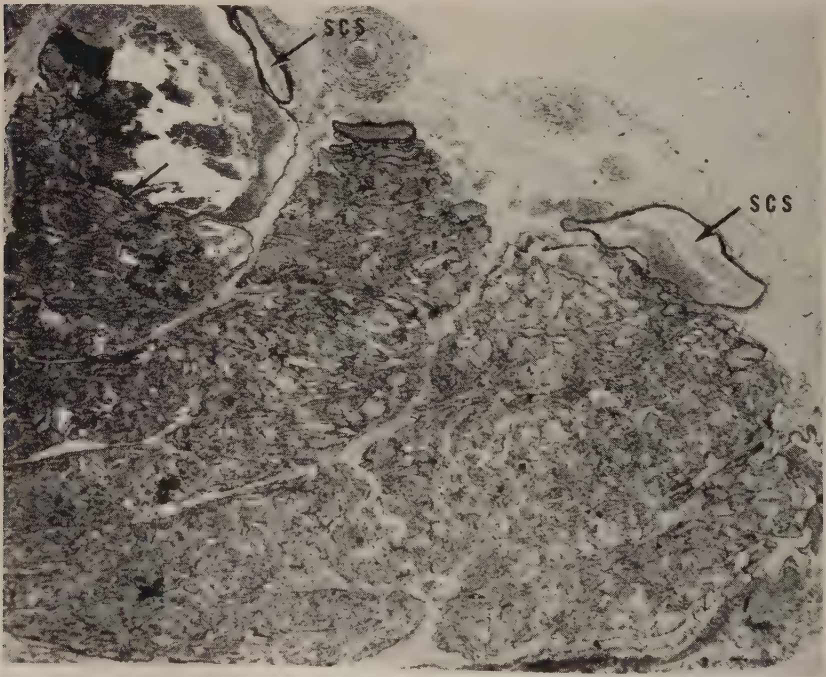

FIGURE 3. Photomicrograph of a section through the ripe placenta of the hyena (*Crocuta crocuta*), showing the chorionic plate and portions of the subchorial sinus (scs). ×20.

extensive subchorial sinus (FIGURE 3). This latter is drained by a series of ostia which communicate with the subjacent intervillous space.

The trophoblastic zone. This zone is composed of a broad layer of placental villi constituting the labyrinth (FIGURE 3). Sooner or later these chorionic villi branch and subdivide, and a number of them form direct anastomoses with corresponding branches of other villi, while others terminate freely. At this stage the villi appear (FIGURE 4) as relatively thin, irregularly branched strands invested by very thin layers of syncytium and provided with an axis of fine fibrillar connective tissue in which are abundant capillaries, often situated immediately below and indenting the syncytial covering. These "intraepithelial capillaries" are a striking feature of the placental villi in *Crocuta* (FIGURE 4). Accordingly, the placenta cannot be described adequately as being formed of a trabecular network; rather, it is a villous placenta in which the greatly branched and relatively slender villi are not completely free but, to a variable and inconstant degree, are connected by direct fusions and syncytial junctions. Some of the main villous stems reach the basal decidua, to which they are attached by their syncytial coverings. Others, on the contrary, penetrate the depth of the intervillous space, but are not attached. In these, the cytotrophoblast survives at the tips of the villi that often occlude the mouths of the uterine glands (FIGURE 5).

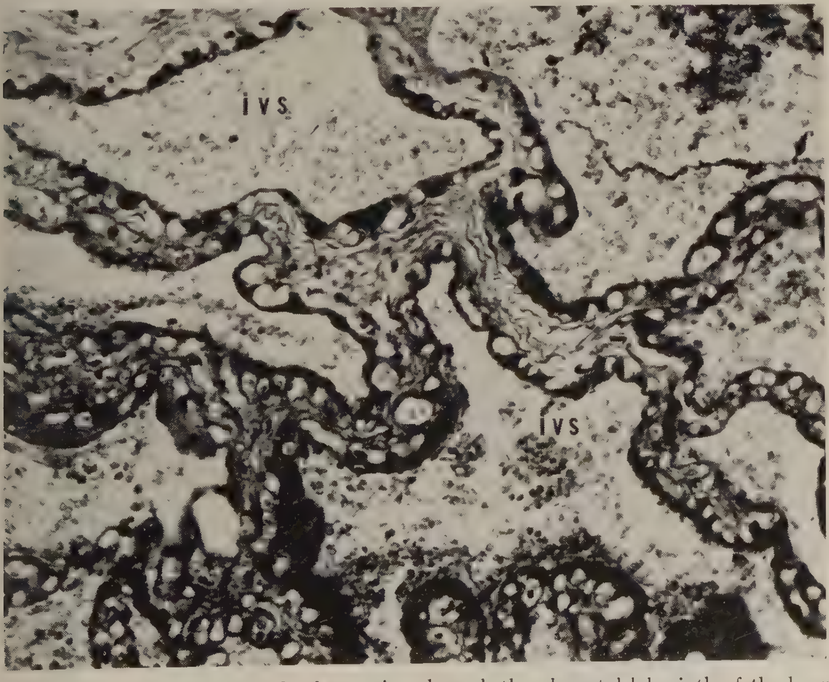

FIGURE 4. Photomicrograph of a section through the placental labyrinth of the hyena, showing the irregularly branching trabeculae invested by very thin layers of syncytium. The "intraepithelial capillaries" are a striking feature of the placental villi. ×220.

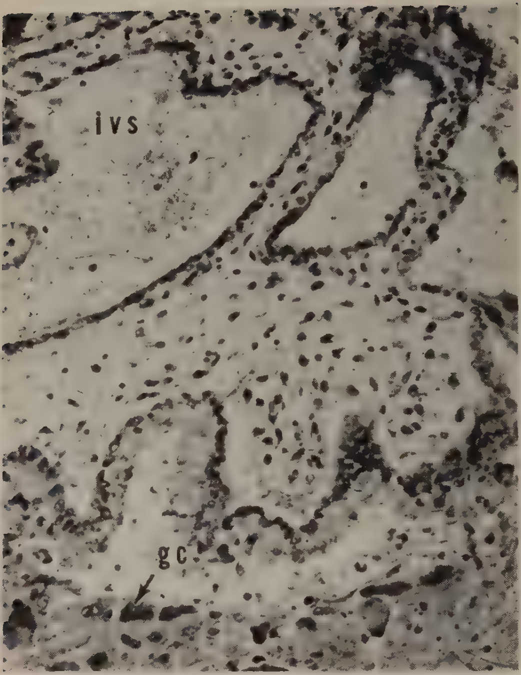

FIGURE 5. Photomicrograph of a section through the tip of a stem villus in the hyena that has penetrated the depth of the intervillous space (ivs). Note the cytotrophoblast that has survived on the crest of the villus, and the giant cells (gc) in the underlying decidua. ×150.

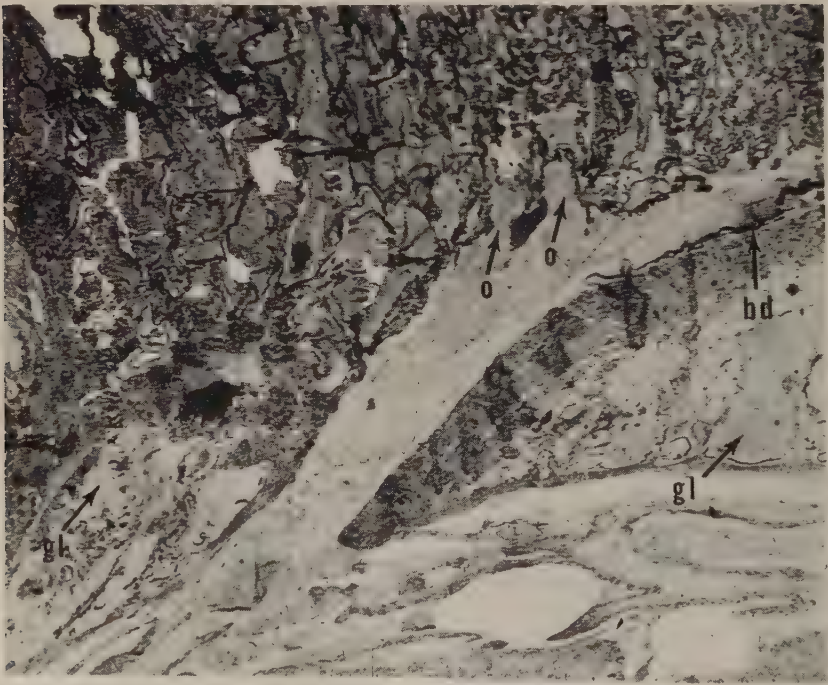

FIGURE 6. Photomicrograph of a section through the base of the placenta in the hyena, showing the manner of exit of the blood from the intervillous space directly into a vein of the basal decidua. ×15.

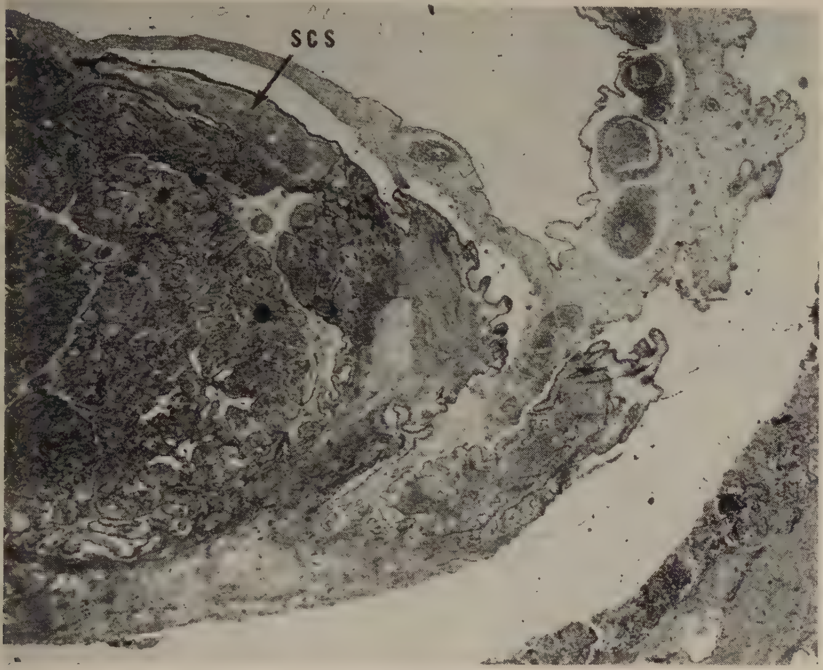

FIGURE 7. Photomicrograph of a section through the margin of the placental band in the hyena. At this point the placenta is undercut by a double layer of allantochorion and has lost contact with the uterine wall, although a discrete robust band of endometrial stroma is carried up along the free edge of the placenta, providing the framework along which the arteries (FIGURE 9) are brought to open into the subchorial sinus (scs). ×8.

The attachment zone. This zone consists of the basal trophoblast, composed of syncytium, which attaches the anchoring villi to the decidua and through which the venous exits occur (FIGURE 6). The endometrium beneath the basal trophoblast shows a marked reaction. The stroma is loosened, somewhat edematous, and fairly cellular. The cells appear for the most part to be enlarged and swollen derivatives of the uterine connective tissue (FIGURE 5). Contiguous to the basal layer of the trophoblast, the maternal cells are undergoing degeneration, as shown by the presence of pyknotic nuclei and cellular debris. These degenerative changes are variable in amount and extent in different locations.

The deep glandular zone. In this zone very few of the uterine glands are visible in the superficial endometrium, or even in the deeper layer of the endometrium (FIGURE 6); although uterine glands can be found with some regularity, they are relatively degenerate and some are filled with blood, recalling the so-called placental sign noted by Hartman (1932).

The marginal zone. The border of the zonary placenta is relatively simple (FIGURE 7); the labyrinth tapers abruptly to form a blunt wedge, from the edge of which the chorion continues as a membrane composed of mesoderm and a

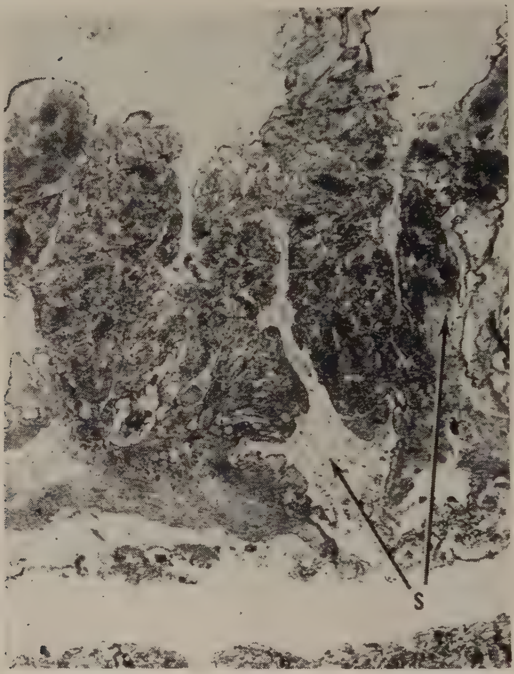

FIGURE 8. Photomicrograph of a section through the placental labyrinth, showing the dividing septa. ×6.

thin layer of trophoblast. The border of the placenta in *Crocuta* is unlike the zonary placenta of carnivores such as the dog and cat in that the formations called border hematoma are entirely lacking (FIGURE 7).

In FIGURE 8 it is obvious that the placental labyrinth is markedly divided by strands or septa of maternal tissue that penetrate the fetal placenta and terminate a short distance below the subchorial sinus. These septa, which are especially well developed at the margins of the placental band (FIGURE 9), convey maternal arteries that terminate in the sinus below the chorionic plate by numerous apertures or fenestrae. FIGURE 9 indicates very beautifully the manner in which an afferent artery rising from the endometrium terminates in the subchorial sinus. In this figure, an ensheathed vessel can be seen coursing obliquely upward along the placental band toward the fetal surface of the placenta. At the level of the photomicrograph the original walls of the vessel have totally disappeared, with the exception of a layer of endothelium that has become discontinuous and patchy. Although this is not demonstrated in a single section, the elongated vessel seen in the photomicrograph communicates widely to right and to left with the subchorial sinus. Thus the arterial blood entering the sinus finally will escape into the intervillous space through the ostia situated at irregular intervals along its placental face.

The venous blood, on the other hand, is returned to the maternal vessels

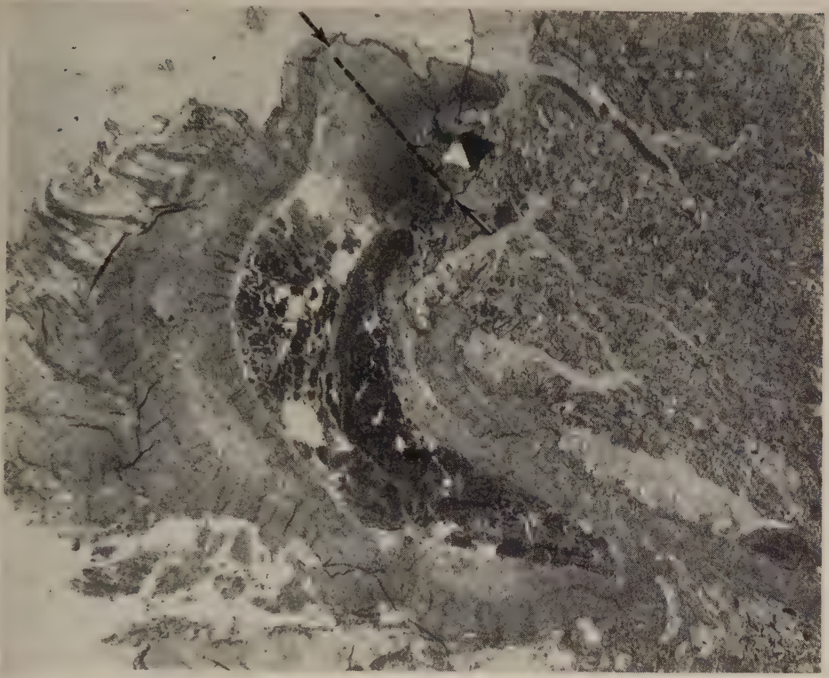

FIGURE 9. Photomicrograph of a section through the margin of a placental band in the hyena, showing the manner in which an afferent artery rising from the subplacental zone terminates in the subchorial sinus. The point at which the artery communicates with the subchorial sinus is indicated by the interrupted line. ×12.

through the base of the placenta, escaping from the intervillous space directly into the veins of the basal decidua (FIGURE 6). Thus, in its main features, namely, the entry of the maternal arteries into the fetal placenta through interlobular septa and the exit of venous blood from the base of the placenta, the circulation in the placenta of the hyena bears, on this score, considerable similarity to the mode of circulation encountered in the placenta of platyrrhine monkeys and in typical labyrinthine placentae, the nature of which in carnivores has been analyzed by Amoroso (1952), and from which the villous hemochorial placenta of *Crocuta* may be derived.

The circulation in the placenta of *Crocuta*, as here outlined, recalls the earlier cotyledonary theory of placental circulation elaborated by Bumm (1893), which admitted basal drainage in the human placenta and envisaged a strictly unitary circulation confined by septal walls in which maternal arteries are distributed. On this view the placental arteries open into the subchorial lake at the summits of the septa or high enough upon them so that arterial blood falling back through the ostia toward the venous outlets in the basal plate bathes the functional villi, thus ensuring the countercurrent flow of the maternal and fetal circulations.

Essentially similar relationships have been demonstrated in the placenta of the hyena. Our observations also revealed very sharply and handsomely the presence of a considerable number of intraepithelial capillaries, the walls of which lie, in places, so near the surface of the placental villi and trabeculae that the syncytium is reduced to a very thin layer. Accordingly, when it is realized that over a considerable area of the placental face only a thin layer of attenuated syncytium, together with a thin plate of endothelial cytoplasm, separate the two blood streams, the intraepithelial capillaries acquire greater significance as a means of exchange between the mother and her offspring.

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ASPECTS OF PLACENTAL FUNCTION

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Introduction

It is a universal biological fact that some provision is made for sustaining the fertilized ovum until it reaches the age at which it can successfully survive as an independent unit. In the animal kingdom the mammal provides this stage by the formation of the placenta. Amoroso[1] has demonstrated that similar provisions for viviparity are made by some submammalian types. This paper confines itself to the intrauterine placenta of the mammal.

It is convenient to visualize that, whereas man has seven ages, as Shakespeare pointed out, the placenta has four ages or stages. These can be called the "implantation age," the "yolk-sac age," and the "early" and "late" ages of the allantoic placenta.

Although this approach may appear somewhat frivolous, it is put forward as a help to understanding the main aspects of placental function. The placenta is a living structure; insofar as we know, it consumes energy at a high rate. It presents a continuous change of shape, structure, weight, and function and, in most species, it grows—in the accepted sense of the word—throughout most of its life. It is peculiar in having a dual origin—in the fertilized ovum, the two embryonic layers, ectoderm and mesoderm, and in the mother, a deciduous layer. The placenta is essential to the development and growth of the fetus until it is able to exist independently (with the qualification of postnatal dependence on the breast).

This paper endeavors to indicate how the placenta fulfills its function. For this reason the first two ages are mentioned only insofar as they affect the last two ages, those involving the actual allantoic placenta, which is, of course, what is meant by the term "placenta" unless it is qualified, particularly as used by the obstetrician and all those interested in the human subject.

FIGURE 1 presents the growth in weight of the fetus in the goat and FIGURE 2, that for the placenta. It will be seen that at about the hundredth day of a 150-day pregnancy the fetal weight first exceeds the placental. This date marks the approximate age at which the goat placenta moves out of the third and into the fourth age of development. In the rat, this happens on the sixteenth day of a pregnancy of 21 days.

The beginning of age 3—the early allantoic placenta. The beginning of age 3 is not synchronous with the termination of age 2, that of the yolk-sac placenta; there is nearly always an overlap. In the rat, the vascularization of the allantoic placenta occurs on the eleventh or twelfth day. This vascularization is an important event in the lifetime of the placenta of all species, marking, as it does, the final union of the component layers of the allantoic placenta. In lower vertebrates such as reptiles and birds, the yolk-sac placenta is at least the main, if not the sole, fetal support and, in many mammals such as the rat,

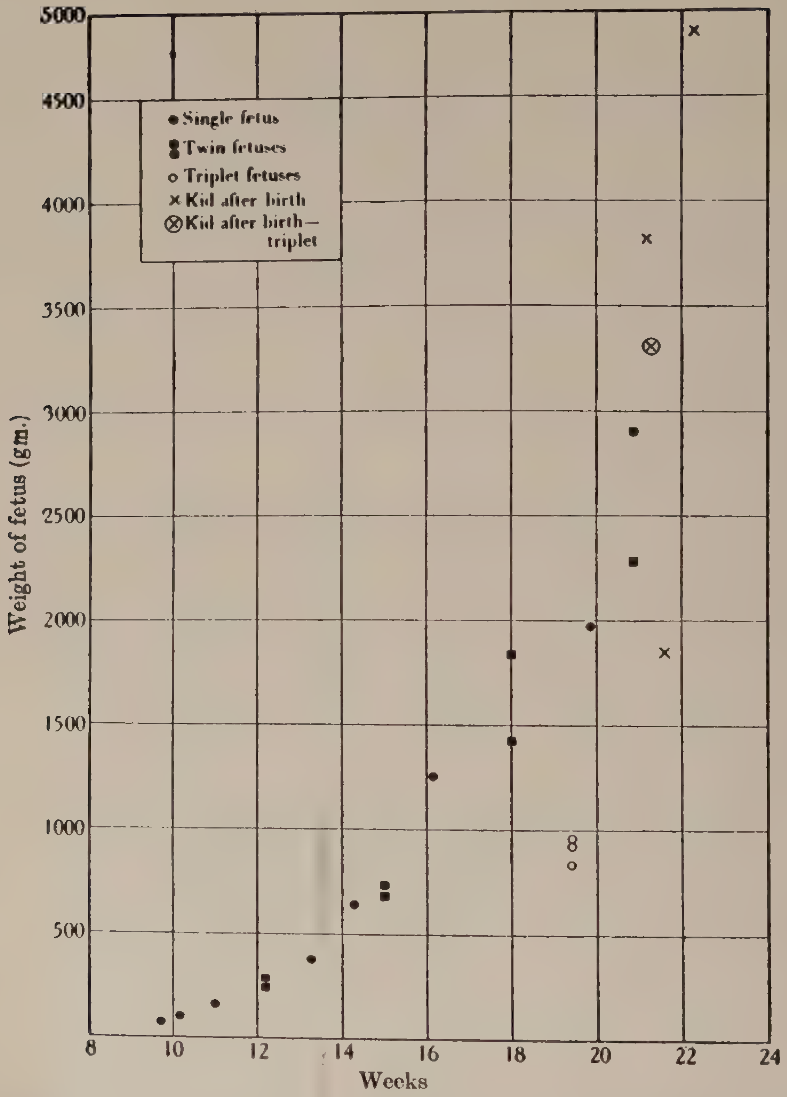

FIGURE 1. Increase in weight of the goat fetus during the 150-day pregnancy. Reproduced by permission from Elliott *et al.*[2]

the yolk sac serves as a real functioning organ preceding the allantoic placenta. In man, however, the yolk-sac placenta vascularizes simultaneously with the villous allantoic placenta, from the sixteenth day, and functions through the fifth to the seventh week, when it atrophies; it is never more than a possible supplementary source of nutrients for the allantoic placenta.

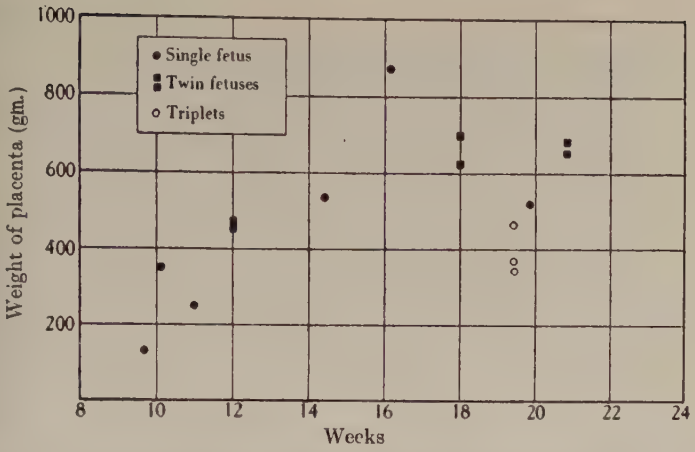

Figure 2. Increase in weight of the placenta with increase in the fetal age of the goat during the 150-day pregnancy. Reproduced by permission from Elliott *et al.*[2]

The ability of the completed allantoic placenta to persist and to lead an existence independent of the embryo was first demonstrated by Giacomini,[3] and Newton[4] showed that after mouse embryos were crushed the placenta persisted to full term when delivered. Van Wagenen and Newton[5] have reported similar findings in the monkey. In all these cases of retention of the placenta after fetal death, the symptoms and signs of "pregnancy" have persisted. These findings have been extended by Huggett and Pritchard[6, 7] and by Pritchard and Huggett.[8]

If the fetus is killed by direct trauma, the blood flow in the allantoic (umbilical) vessels stops, and the growth of the allantoic vessels and of the mesodermal mesenchyme ceases. If this occurs in late fetal life, then the formed placenta appears to retain the powers of differentiation and growth that are resident in the trophoblast and decidua. Such a placenta can be carried to full term with delivery, or aborted before reaching term, or retained. However, if fetal death is caused before the mesoderm and its vessels infiltrate the trophoblastic villi (in villous types such as man) or the labyrinth and spongy zone (in labyrinthine types such as the rodent), or while infiltration continues and is still incomplete, then the outcome is somewhat different. Very soon, if not immediately, the ectodermal trophoblast ceases mitosis and growth. The mesoderm and allantoic mesenchyme necrosis may form a blood clot, and the placenta as a whole regresses, forming a compact mass that usually aborts and diminishes to a scar or clot. The placenta may persist without going through labor or abortion. If so, it shows resemblances to the human benign mole in which organization of the fetal vasculature is absent or minimal, the allantoic mesenchyme is necrotic and edematous, and only the trophoblast of the villi persists independently. This difference, which is due to age at death, is apparently largely mechanical—the result of the collapse of the imperfectly formed placenta and its villi, rather than an intrinsic primary function of the mesoderm.

FIGURES 3, 4, and 5 show the progressive disappearance of the placenta at the fifteenth and nineteenth days, after fetal death on the tenth day. FIGURES 6, 7, and 8, with a larger magnification, contrast the development at the fifteenth day of the placenta when fetal death has taken place on the tenth day, the mesoderm barely having reached the trophoblast, and on the twelfth day, the mesoderm having reached the trophoblast. The latter shows far more critical differentiation, in contrast to the former, where detail is blurred by edema and diffuseness, or nonexistence. This age at which mesoderm invades ectoderm is critical. Chesley,[9] for instance, found that fetal mice homozygotes for "short tail" (Brachyura) failed to form mesoderm and, therefore, failed to vascularize their placentas. Death occurred at the eleventh day and was followed by resorption. In addition, Evans and Burr[10] showed that in rats a vitamin E deficiency stops vascularization by the allantoic vessels, and that fetal death ensues; similarly, Hammond[11] has shown that in rats spontaneous death occurs at the twelfth day (equivalent to the eleventh in mice), and that in such cases a lethal gene may be involved. Furthermore, necrosis of the decidua is caused in rats before the twelfth day by estrone, gonadotrophin, and ovariectomy. After that date they are no longer lethal to the fetus, and gonadotrophin, pregnant mare serum, can even prolong pregnancy (Huggett and Pritchard[7]). Ewart[12] has shown that spontaneous abortion in horses is most frequent at the seventh week, when the yolk sac gives way to the allantoic placenta. Humans, as previously mentioned, exhibit an analogous obstetric pathology, whereby, when the fetus has died, a "placenta" persists, forming a mole of the fleshy nonmalignant, the hemorrhagic, or the cystic type. In all these cases a mesodermal layer has formed on the placenta, as discussed by Adami[13] and Novak,[14] but formed vessels are minimal and whatever mesenchyme is present is edematous and degenerate.

The early and late ages of the allantoic placenta. The basis for dividing the life of the allantoic placenta into two periods, early and late, is, first, that in the later period the rate of fetal reception of nutrients across the placenta is of an order different from that in the first period. In man, an average of approximately 8 gm. a day pass across the placenta during the first 6 months, but this increases to about 26 gm. a day during the last 3 months, of which 36 gm. a day pass in the last month. Second, the placenta either slows up its rate of growth or loses weight. Third, even when it continues growth to full term, as in man (Adair and Thelander[15]), it shows signs of necrosis or degeneration, although it is transmitting nutrients better than ever. In the normal rat placenta, there is a region of necrosis and leukocyte infiltration at the placental margin; in man, calcification of arteries and infarction with consequent necrosis are visible. It has been said that it is physiological for the placenta to be pathological. Finally, in this later period some functions show a decrease in activity, whereas others increase up until labor itself, or even further, in delayed labor or prolonged gestation.

The control of placental development. Clearly it would be of interest to be able to determine placental development and improve it where it is defective. Philosophically it would be advantageous to know the factors involved in its growth,

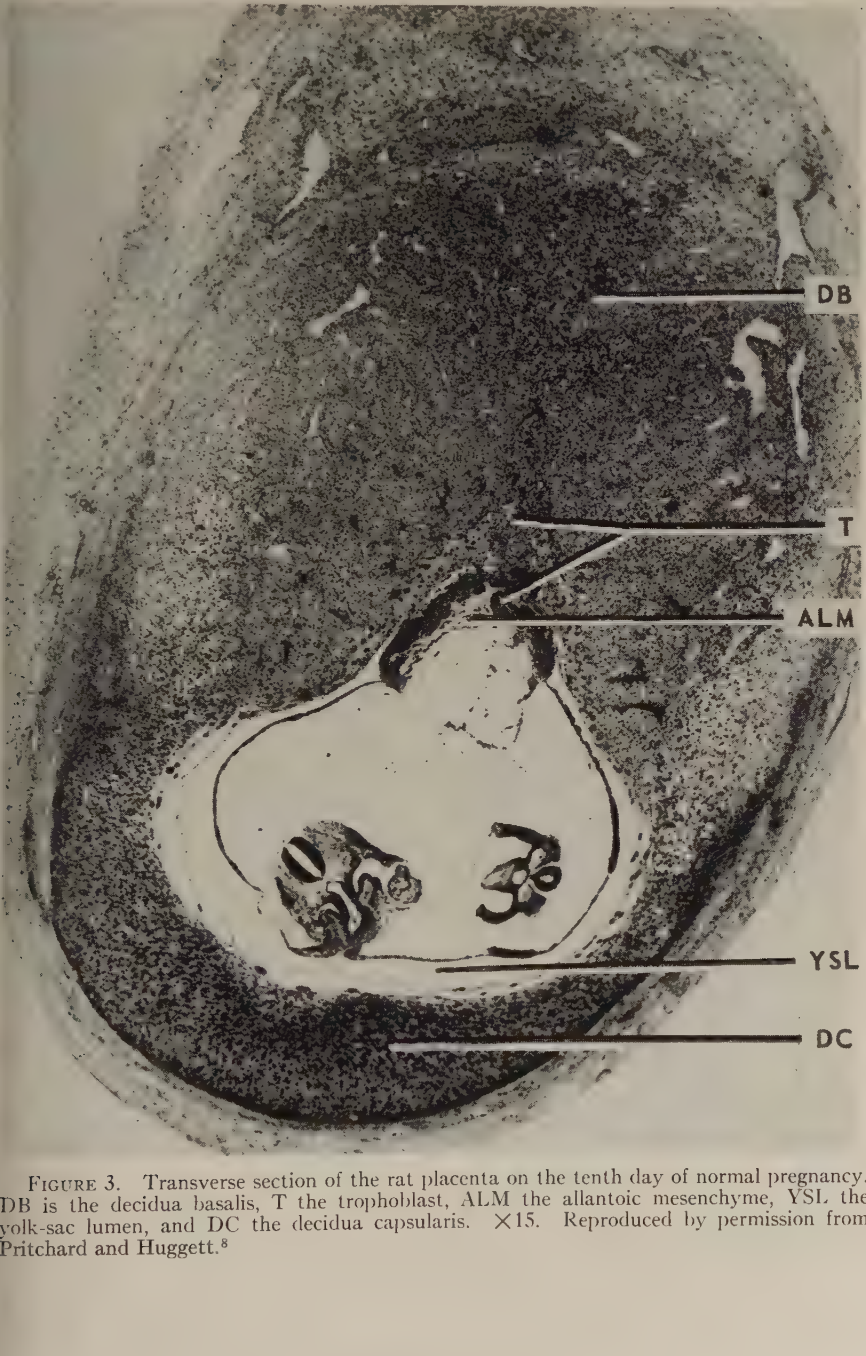

FIGURE 3. Transverse section of the rat placenta on the tenth day of normal pregnancy. DB is the decidua basalis, T the trophoblast, ALM the allantoic mesenchyme, YSL the yolk-sac lumen, and DC the decidua capsularis. ×15. Reproduced by permission from Pritchard and Huggett.[8]

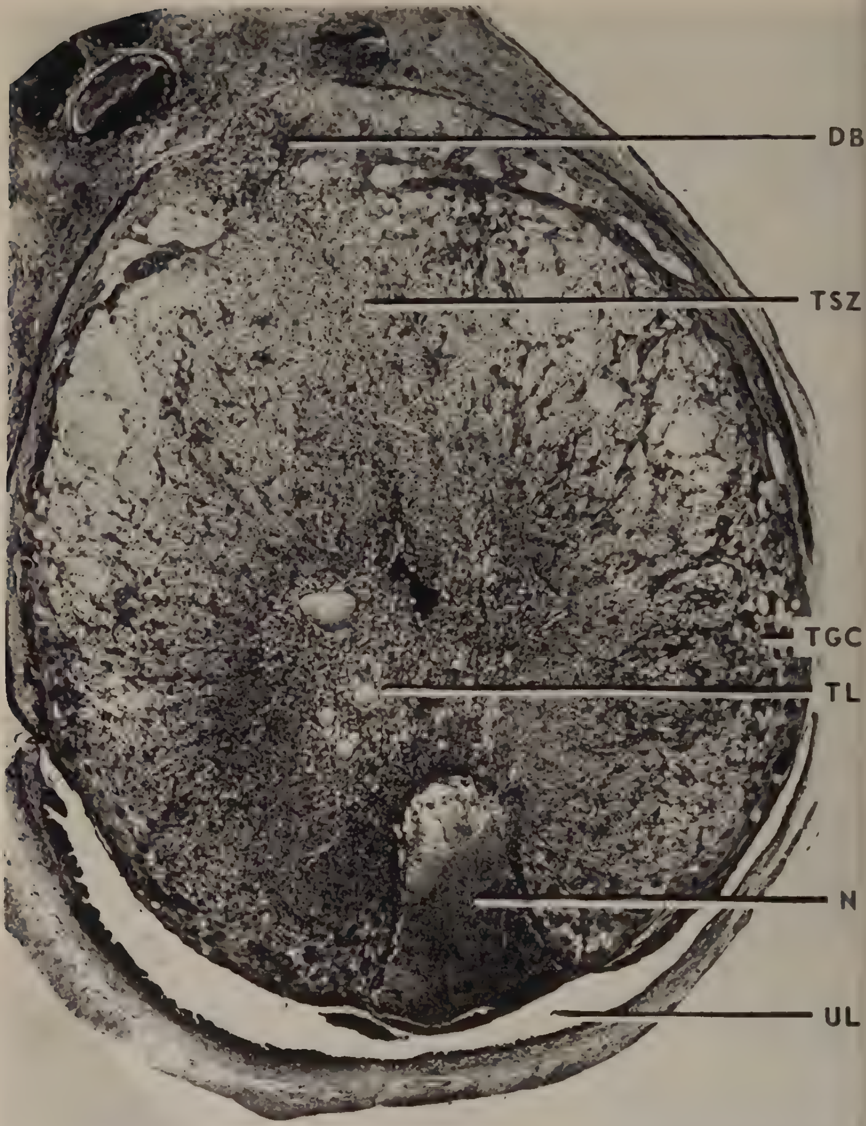

FIGURE 4. Transverse section of the rat placenta on the fifteenth day, the fetus having been killed on the tenth day. DB is the decidua basalis, TSZ the trophoblast spongy zone, TGC the trophoblast giant cells, TL the trophoblast labyrinth, N the necrotic mass, and UL the uterine lumen. ×15. Reproduced by permission from Pritchard and Huggett.[8]

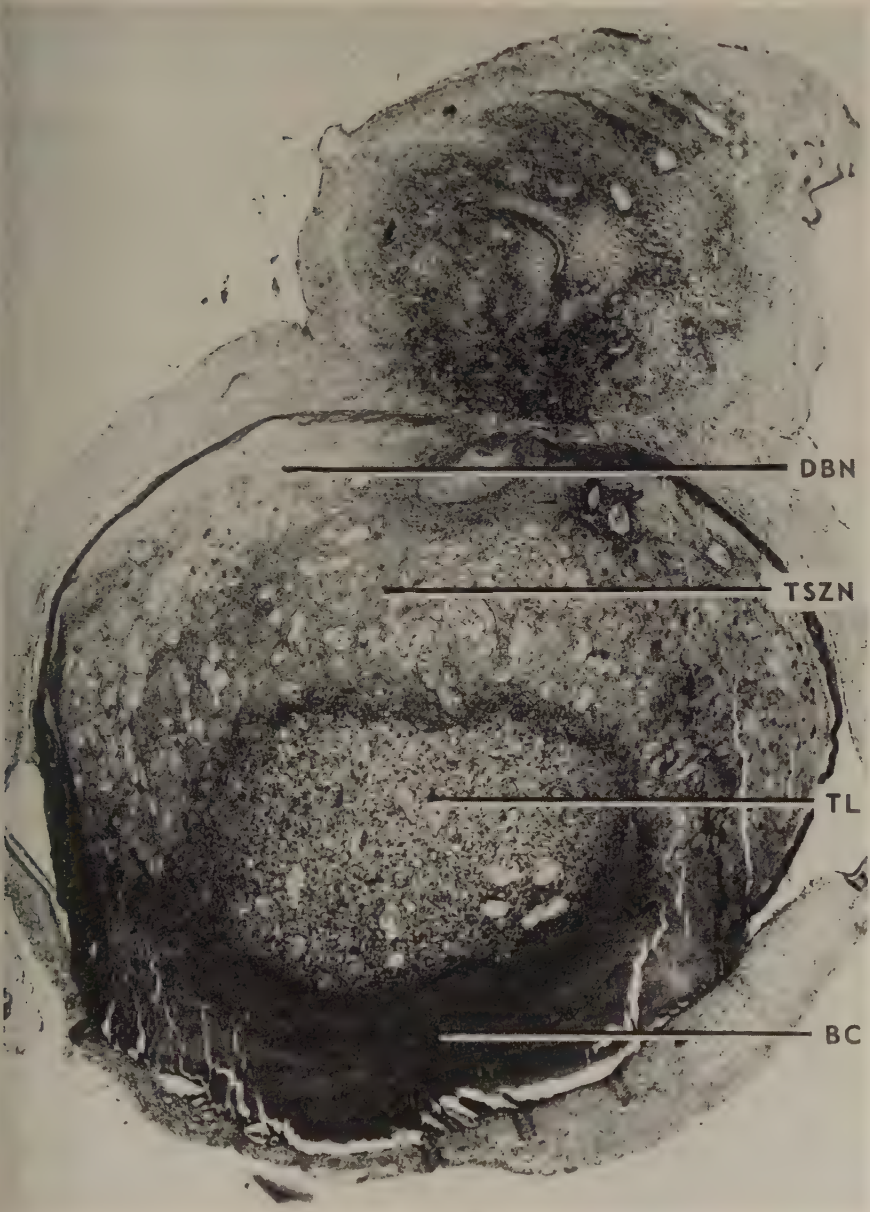

Figure 5. Transverse section of the rat placenta on the fifteenth day, the fetus having been killed on the tenth day. DBN is the necrotic decidua basalis, TSZN the necrotic spongy zone of the trophoblast, TL the trophoblast labyrinth, and BC the blood clot. ×15. Reproduced by permission from Pritchard and Huggett.[8]

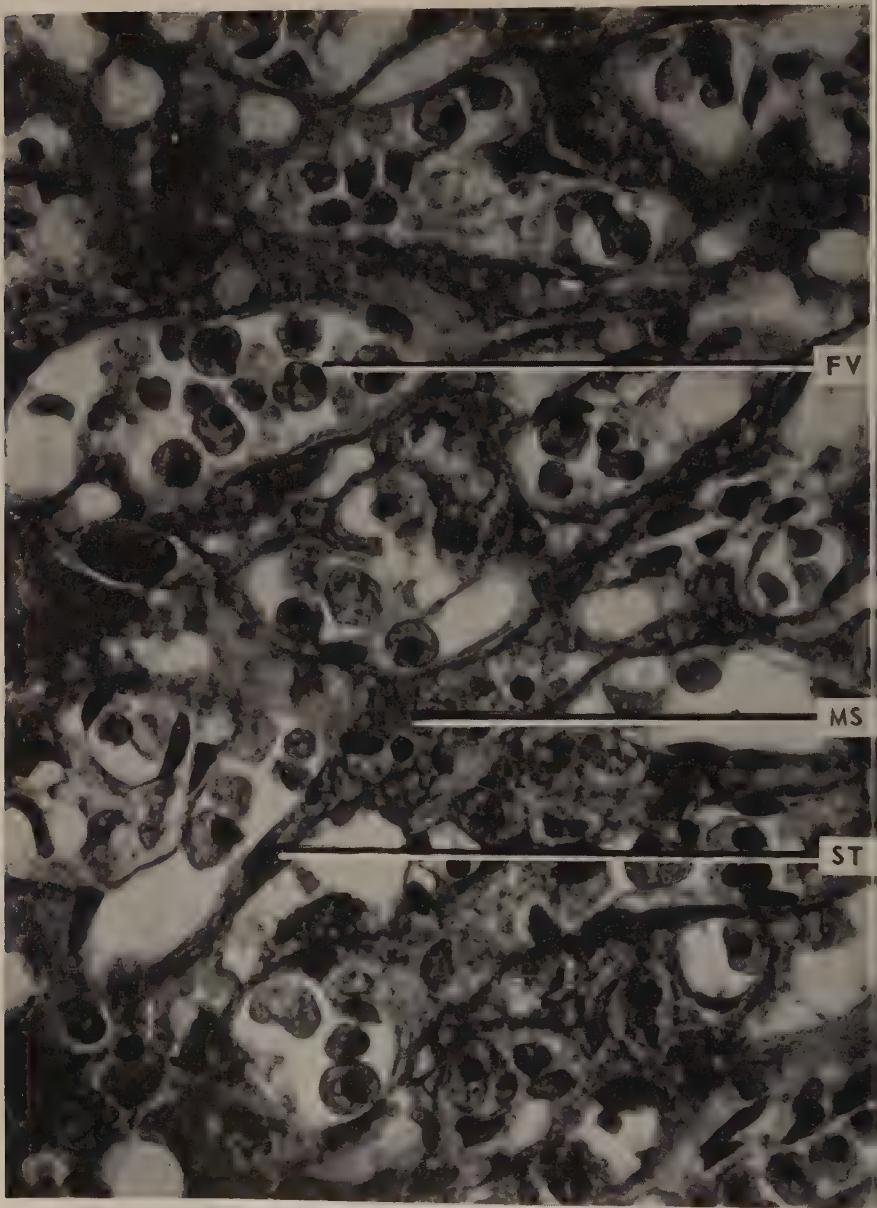

Figure 6. Normal labyrinth of the rat placenta on the fifteenth day of pregnancy. FV is the fetal vessel, MS the maternal sinus, and ST the syncytiotrophoblast. ×392. Reproduced by permission from Pritchard and Huggett.[8]

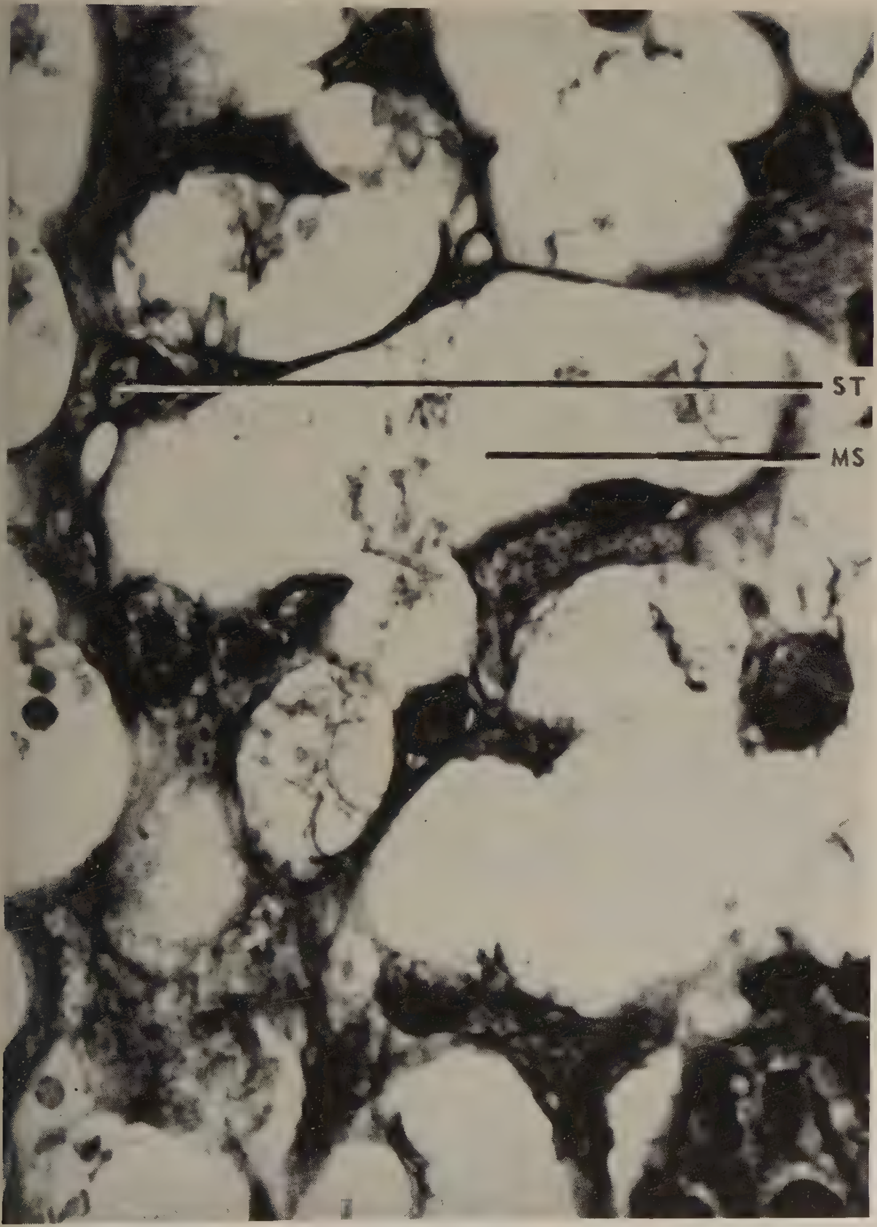

Figure 7. Labyrinth of the rat placenta on the fifteenth day of pregnancy, the fetus having been killed on the tenth day. ST is the syncytiotrophoblast and MS the maternal sinus. ×392. Reproduced by permission from Pritchard and Huggett.[8]

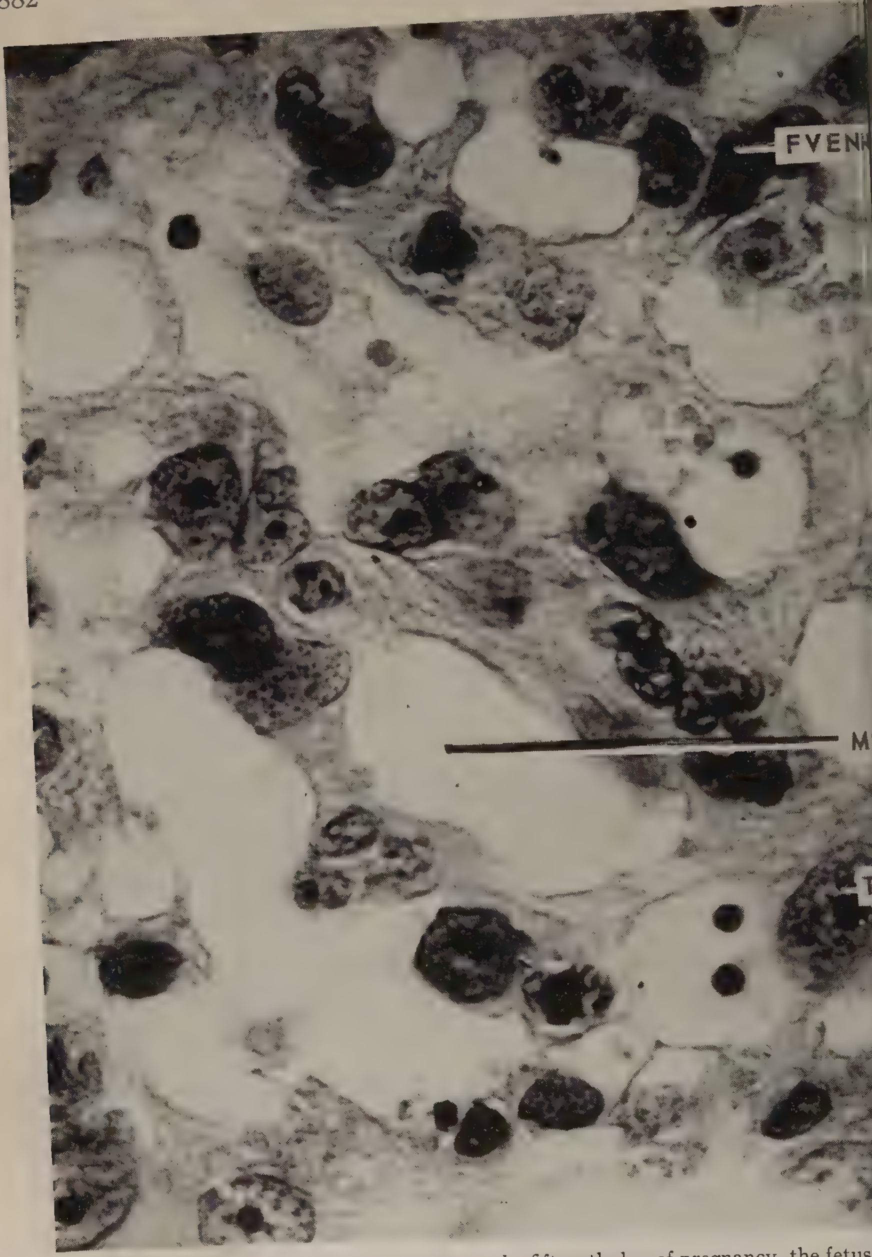

FIGURE 8. Labyrinth of the rat placenta on the fifteenth day of pregnancy, the fetus h ing been killed on the twelfth day. FVEN is the fetal vessel endothelial nucleus, MS maternal sinus, and TN the trophoblastic nucleus. ×392. Reproduced by permis from Pritchard and Huggett.

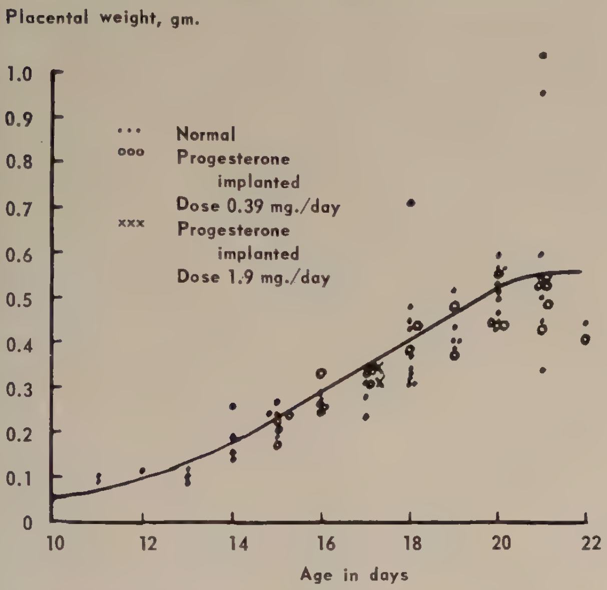

FIGURE 9. Placental weight changes in the rat at various fetal ages, showing the effects of progesterone.

differentiation, and control. This knowledge, it is hoped, would then be available for improvement of the placental function and the greater health of the fetus *in utero*, as well as that of the newborn infant.

We start with the negative fact that the nervous system appears to exert no direct control over the placental development. Amoroso[16] has described nerve fibers in the umbilical cord, but no function has been attributed to them.

Progesterone, whether implanted or injected into pregnant rats, seems to cause a fall in placental weight in a large percentage of cases (FIGURE 9), an increase in fetal weight (FIGURE 10), and a consequent rise in the P/F (placental to fetal) ratio (FIGURE 11) and the log P/F (FIGURE 12). There is also a greater fall in the placental glycogen than is normally found (FIGURE 13). Progesterone appears to accelerate or advance both placental and fetal development.

The figure shows that there are individual points that are not in accordance with the general proposition or the average result. This is due, of course, to the fact that other factors are involved. Small doses of estrogens appear to stabilize, but larger doses terminate, pregnancy. Relaxin has been shown by Frieden and Velardo[17] to terminate pregnancy. In my experiments it showed itself to be detrimental to fetal growth *in utero*.*

In a series of pregnant rats given daily doses of 1 mg. of progesterone, 1.5 µg. of estradiol, and 30 G.P.U. (guinea pig units) of Releasin either alone or in

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* Robert L. Kroc of Warner-Chilcott Laboratories, Morris Plains, N. J., generously provided samples of Releasin for my experiments.

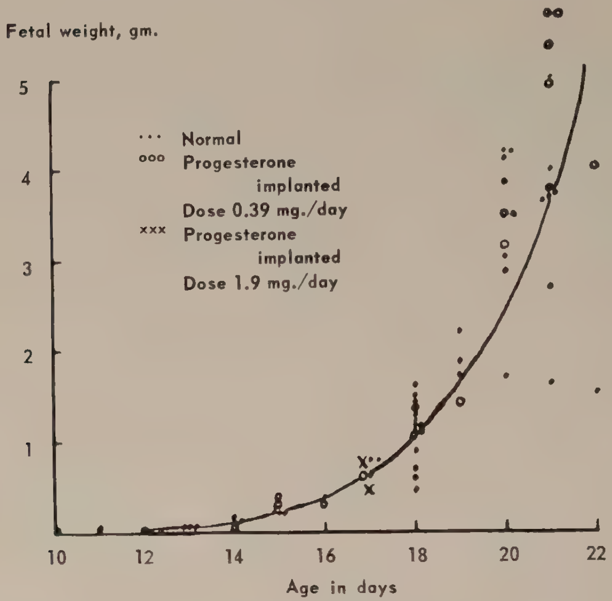

FIGURE 10. Fetal weight changes in the rat at the various fetal ages for which placental weights are given in FIGURE 9, showing the effects of progesterone.

combination, the percentage of live births obtained by Muriel Jones and myself[18] was maximal for those receiving progesterone alone, 100 per cent; 50 per cent for estradiol; none for Releasin; 100 per cent for progesterone and estradiol in combination; 59 per cent for progesterone and Releasin in combination; 33 per cent for estrogen and Releasin in combination; and 30 per cent for all three together

Velardo,[19] too, has shown that adrenocorticotrophin also terminates pregnancy, whereas Hoet[20] claimed that placental glycogen is increased by 0.5 mg./kg. of cortisone, but that 2 mg. terminated pregnancy. This is important, since the glycogen in the placenta appears to have special functions.

Put briefly, it is clear that further work is needed on this problem with respect to both the fetal and the maternal hormones. In Paris Jost has already shown by some beautiful work that the fetal endocrine system is far from negligible; in fact, it is a major factor in the production of normal births, although its precise role, if any, in growing pinker and plumper placentas is not yet known.

The function of the placenta. In conclusion it is appropriate to analyze the functions of the placenta in the light of modern knowledge. First, there are specific placental activities that proceed unimpeded to a successful career ending with maximal powers at the height of intrauterine life. Such activities include the passage of nutrients and the removal of fetal metabolites, and probably also the secretion of hormones to the mother to replace or supplement her own, namely, progesterone, estrogens, and gonadotrophins. Here it is necessary to emphasize the work of Amoroso,[16] who has shown that viviparity depends on the power to avert the next estrus, and that this is developed

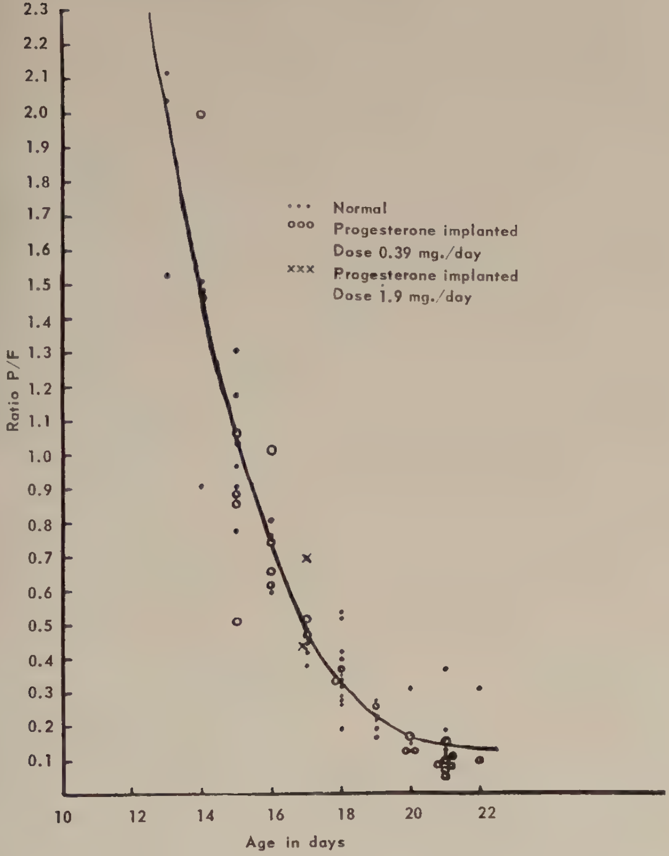

FIGURE 11. Ratio of placental and fetal (P/F) weights for FIGURES 9 and 10.

in the vertebrate and the mammal by the placenta's secretion of gonadotrophins and ovarian hormones. This is a major contribution to the philosophy of the placenta.

Second, one should emphasize that it is doubtful whether anything passes across the placenta by gradient physical diffusion, except, possibly, gases. All the evidence shows that, even for water and salts, and certainly for sugars and amino acids, transfer is either active or facilitated, involving energy consumption by the placenta. Reversing a current by reversing a gradient probably only involves reversing the position of the greater active mass at the end of a chain of reactions.

Third, one would emphasize that some activities of age 3, that of the early placenta, wane in age 4 *pari passu* with the growth of the fetus. These are shown in TABLE 1, which provides the dates of maximal activity of some of the placental functions. The list does not pretend to be exhaustive. In fact, it is restricted rather to some of the functions that specifically interest me. The actions are largely metabolic, which suggests that there is more than a grain

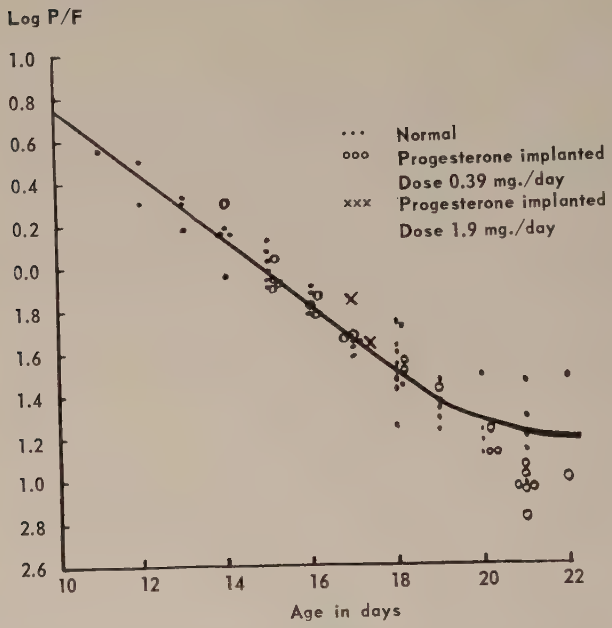

FIGURE 12. Logarithm of the ratio of placental and fetal (P/F) weights given in FIG-URE 11.

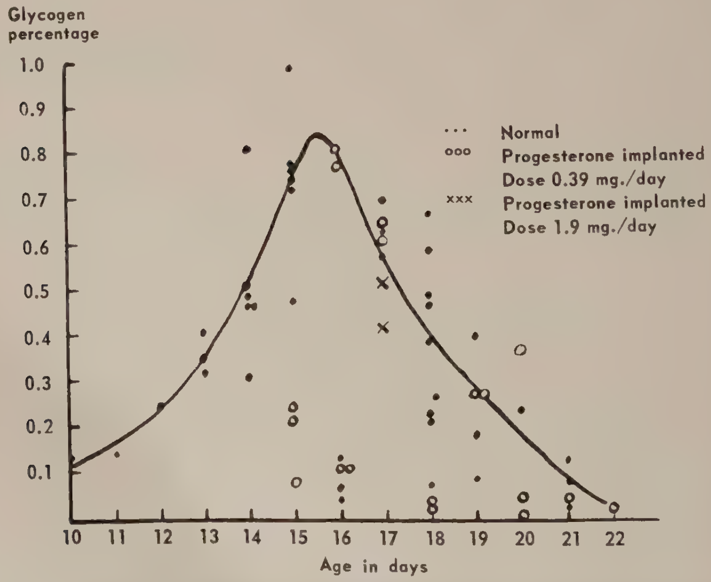

FIGURE 13. Effects of progesterone on glycogen concentration in the placenta of the ra at various ages.

TABLE 1*
PRETERM MAXIMA

Animal and substance	Fetal age at maximum	Concentration mg. per cent
Rabbit		
Phospholipin	30	1500†
Free cholesterol	30	275†
Cholesterol ester	21	400†
Neutral fat	17	970†
Glycogen (fetal)	14	1540‡
Glycogen (maternal)	21	5570‡
Rat		
Glycogen (whole placenta)	16	830§

* Full-term: for rabbits, 30 days; for rats, 21 days.
† Boyd.[21]
‡ Lochhead and Cramer.[22]
§ Huggett.[23]

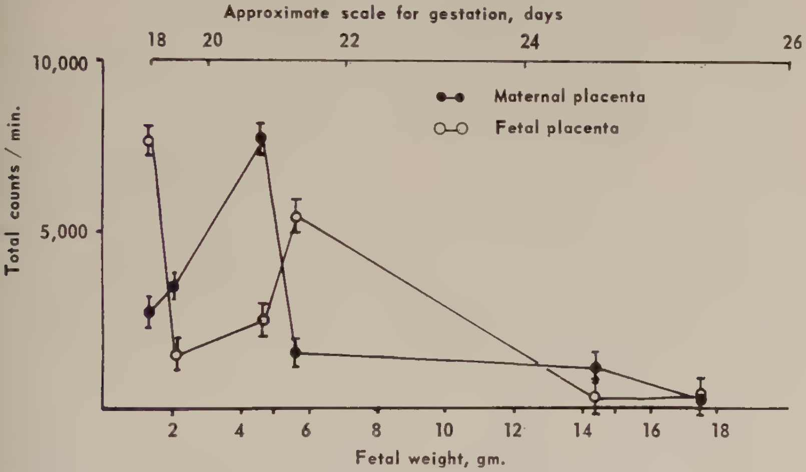

FIGURE 14. The incorporation of labeled glucose as labeled glycogen in the decidual and trophoblastic glycogen deposits of the placenta in the rabbit at various fetal ages. Reproduced by permission from Huggett and Morrison.[24]

of truth in Bernard's generalization that the placenta is the liver of the fetus. FIGURE 14 shows the results of some studies conducted by Huggett and Morrison,[24] which indicate that radioactive glucose becomes incorporated in the placental glycogen at different ages depending on the area of deposition, trophoblastic and decidual, and that both diminish markedly before full term.

Finally, there is good reason to believe that there are hazards and emergencies for intrauterine life. These are by no means absent, and those of us who are alive today are merely survivors because of adequate placentas rather than because we are "the fittest." In coping with these hazards, which include such worries to the fetus as a rise in the cost of living measured by the supply and availability of such currency as oxygen, the placenta can cooperate with the fetus. In some unpublished work by June Barker of Duke University, Durham, N. C., and my colleagues in London on hypoxia in fetal sheep, it has been shown that at rest the fetus and placenta are either in equilibrium, or that the fetus is in a better state of aerobiosis than is the placenta, and so receives lactic acid from it. However, when hypoxia was produced, the fetus was able to rid itself of its lactic acid because the placenta absorbed it. To what extent this happens with other substances in other hazards is unknown to us, but all our evidence indicates that the placenta is a thoughtful organ treating its younger and later developing twin with due regard and consideration.

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PLACENTAL STEROIDS

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Placental Hormones

Although the first steroid hormone isolated from human urine was estrone,* a placental hormone, there is still considerable doubt about the exact nature of the steroids secreted by the placenta. In fact, in comparison with the other steroid-producing glands, relatively little is known about the steroids secreted by the placenta.

In TABLE 1 are listed the fifteen steroids that have been isolated or sufficiently characterized in human placental tissue. Some of these are clearly steroid metabolites and not secretory products. This is certainly true for compounds such as pregnanediol, allopregnanolone, allopregnandiol, and tetrahydrocortisone, which are saturated compounds and have been isolated in such small quantities from placental tissue that their presence can best be explained by assuming that they were actually present in the blood trapped in the placental sinuses. The isolation of hydrocortisone, cortisone, and the other steroids at the bottom of the table deserves further comment. Although there is indirect evidence for the secretion of adrenocortical-like hormones by the placenta, the evidence is predominantly clinical. The frequent improvement of the Addisonian patient during pregnancy, for example, has been used as evidence for the secretion of adrenocortical-like hormones by the placenta. The finding by Jailer and Knowlton[9] of substances in the placenta that give color reactions similar to those of the adrenocortical hormones and the demonstration by Johnson and Haines[10] of hydrocortisonelike activity in placental tissue give considerable support to this hypothesis. However, since the blood trapped in the placental sinuses accounts for approximately 9 per cent of the placental weight,[11] and since the levels of the corticosteroids in maternal blood are known to be elevated, it is possible that the small amounts of corticosteroids detectable in placental tissue can be accounted for by assuming that they are derived from contaminating blood. Salhanick *et al.*[11] calculated the placenta-maternal blood gradient for some of the steroids isolated from placental tissue. This gradient for estrogens is approximately 100 to 1, for progesterone 20 to 1, but for corticosteroids less than 3 to 1. These investigators also conclude that the presence of corticosteroids in the placenta could be due to contaminating blood.

There is at present no definitive answer to the problem of the secretion of corticosteroids by the placenta. The improvement often seen in Addisonian patients during pregnancy may be based on mechanisms not under the control of the adrenocortical hormones. Finally, it is important to add that no corticosteroid or metabolite of a corticosteroid has been identified in either the urine or blood of a pregnant Addisonian whose replacement therapy has been stopped.

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* Throughout the text "trivial" names rather than exact chemical names are used.

TABLE 1

STEROIDS ISOLATED FROM HUMAN PLACENTAS

Estrone,[1] estradiol,[2] estriol[3]
Progesterone[4, 5]
20-α-Hydroxyprogesterone[6]
20-β-Hydroxyprogesterone[6]
Allopregnan-3-β-ol-20-one[7]
Pregnan-3α-20-α-diol[7]
Allopregnan-3β-20-α-diol[7]
Cortisone,[8] hydrocortisone[8]
Reichstein's compound U[8]
11-Dehydrocorticosterone[8]
Aldosterone[8]
Tetrahydrocortisone[8]

TABLE 2

ESTROGENS ISOLATED FROM HUMAN PLACENTAS

Estradiol, 51 γ/kg.[12]
Estrone, 170 γ/kg.[12]
Estriol, 314 γ/kg.[12]
Daily production approximately 100 mg.[13]
Turnover time 6 min.[13]

TABLE 3

PROGESTOGENS ISOLATED FROM HUMAN PLACENTAS

Progesterone 700 γ/full-term placenta	
20-β-Hydroxyprogesterone 20-α-Hydroxyprogesterone	approximately 100[15]
Daily production approximately 250 mg.[13]	
Turnover time 3 min.[13]	

Three estrogens have been isolated from placental tissue: estradiol[2] estrone, and estriol.[3] Some pertinent figures about estrogen secretion by the placenta are shown in TABLE 2. There is little doubt that estradiol and estrone are secretory products; estriol is usually considered to be a metabolite. However there is evidence that estriol may be secreted by the placenta as well.[14] I is likely that the daily production of estrogens by the placenta is approximately 100 mg.[13] The turnover time (the time within which the total bloo pool of estrogens is renewed) is astonishingly brief—6 min.[13] This time i somewhat longer than the turnover time of progesterone but much shorte than that of hydrocortisone, which is approximately 5 times as long.

TABLE 3 lists the progestational substances isolated from placental tissue The 20α- and the 20β-hydroxyprogesterones recently were isolated from placental tissue by Zander *et al.*,[6] who found the same compounds in pregnanc blood and corpora lutea. These compounds are isomers, and the difference between them and progesterone lies merely in the reduction of the ketone group in position 20. The ratio of the concentration of progesterone to these hydroxyprogesterones in placental tissue is approximately 7 to 1. In the Hooker-Forbes test, which is a very sensitive assay for progesterone activity, the 20β-hydroxyprogesterone compound is almost twice as active as progesterone, whereas the 20α isomer is slightly less active than progesterone. The biological significance of these compounds is still unknown.

TABLE 3 lists also the quantities of secreted progesterone. Approximately 700 μg. of progesterone has been isolated from 1 full-term placenta. The daily production is approximately 250 mg., and the turnover time has been calculated by Pearlman[13] to be about 3 min. Every 3 min., therefore, the total progesterone pool is replaced. This confirms earlier experiments, which demonstrated the rapid disappearance of progesterone from circulation. Recently, Pearlman has shown that the metabolism of progesterone in pregnancy is no different from its metabolism in the nonpregnant state.[16]

Biogenesis of the Placental Hormones

In vitro studies of the biogenesis of the steroid hormones have increased greatly our understanding of the physiopathology of the steroid-producing glands. For this reason, a brief discussion of the biogenesis of the placental hormones will be given. In comparison to what we know about the biogenesis of the hormones of the adrenal, the testis, and the ovary, little is known about the biogenesis of the placental hormones. From the available evidence, however, it can be concluded that the pathways of synthesis in the placenta are essentially the same as the pathways of synthesis in the ovary, the testis, and the adrenal.

FIGURE 1 shows a composite picture of most of the enzymatic reactions involved in the biogenesis of the steroid hormones. In the adrenal, progesterone is the key compound. By hydroxylation in position 17 and in positions 11 and 21, progesterone can be converted into hydrocortisone. If hydroxylation occurs in positions 21 and 11, but is omitted in position 17, progesterone then will be converted into corticosterone and possibly aldosterone. Part of the 17-hydroxyprogesterone moiety, however, may not undergo further hydroxylation; instead, the side chain is eliminated, resulting in the formation of C-19 androgens. Finally, these may be converted to estrogens.[17] In the normal adrenal this latter pathway, resulting in the formation of androgens and estrogens, seems to be of minor significance. Recent evidence indicates, however, that it is the major pathway of synthesis of the androgens in the testis,[18] and of estrogens in the ovary.[19] FIGURE 2 indicates the known intermediates between cholesterol and the estrogens in the ovary.[19]

Most of the reactions known to play a role in the synthesis of the estrogens by the ovary have been demonstrated in the placenta, as well. The presence a 3-β-ol-dehydrogenase, the enzyme catalyzing the conversion of pregnenolone to progesterone, was reported by Samuels *et al.*[20] The conversion of cholesterol to progesterone and the intermediacy of pregnenolone in this reaction was shown by Solomon *et al.*[21] The conversion of progesterone to 17-hydroxyprogesterone has not been reported yet, but its existence can be inferred from the isolation of pregnantriol (a metabolite of 17-hydroxyprogesterone) from

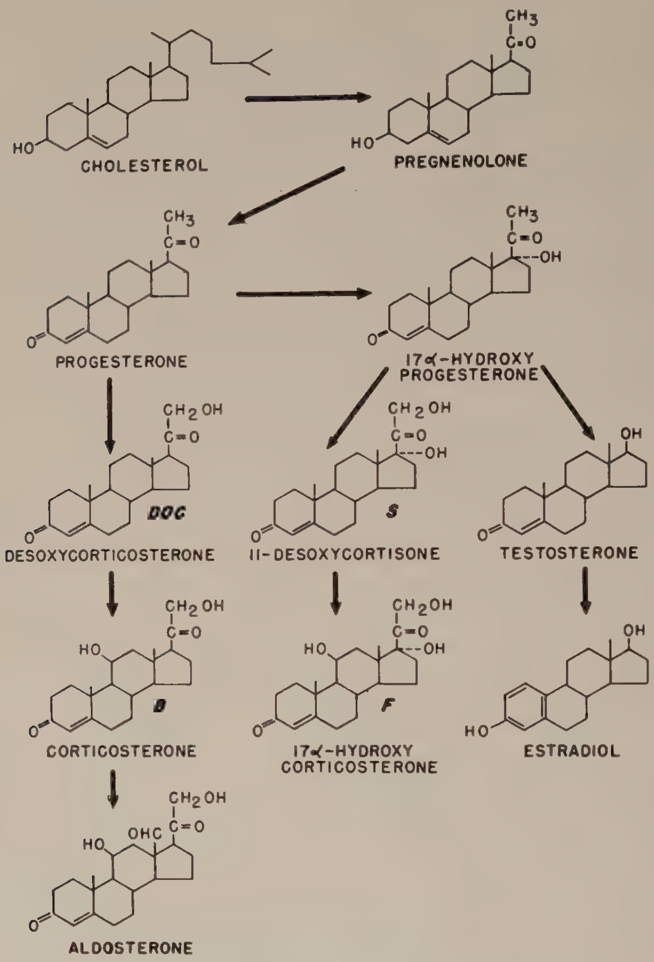

FIGURE 1. Biosynthesis of the steroids from cholesterol.

the urine of a pregnant, bilaterally adrenalectomized patient.[22] An important missing link in the sequence is the lack of a demonstration of the conversion of progesterone to testosterone. The conversion of androstenedione to estrone was shown by Ryan,[23] and the conversion of 19-hydroxyandrostenedione to estrogens by Meyer.[24] Therefore, although the evidence is still incomplete, it seems likely that the synthesis of the estrogens in the placenta follows the same pathways as the synthesis of the estrogens in the adrenal, the testis, and the ovary.

In addition to the conversions discussed above, the conversion of progesterone to 6-keto-progesterone has been shown in placental perfusates,[25] and its conversion to 6-β-hydroxyprogesterone in placental incubates (FIGURE 3).[26] 6-β-Hy-

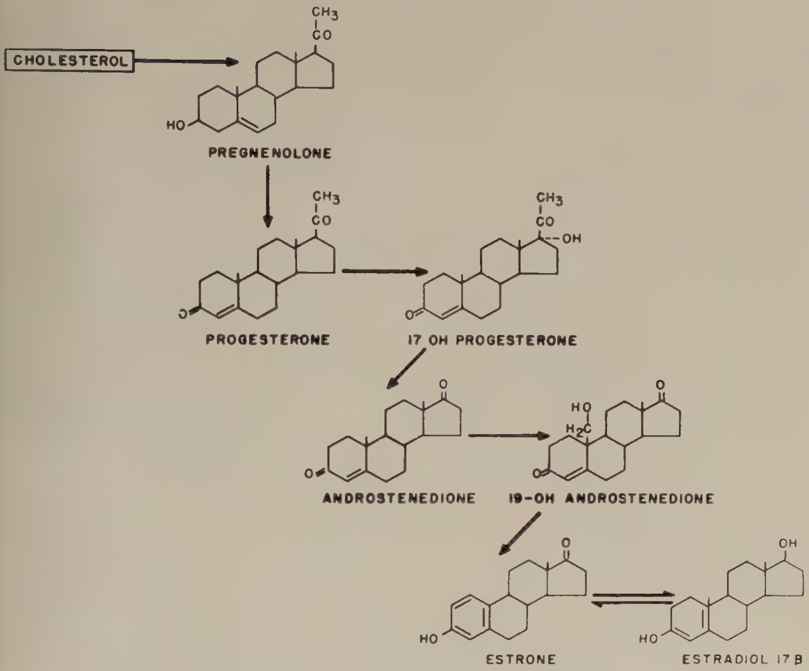

FIGURE 2. Biosynthesis of the estrogens by the ovary.

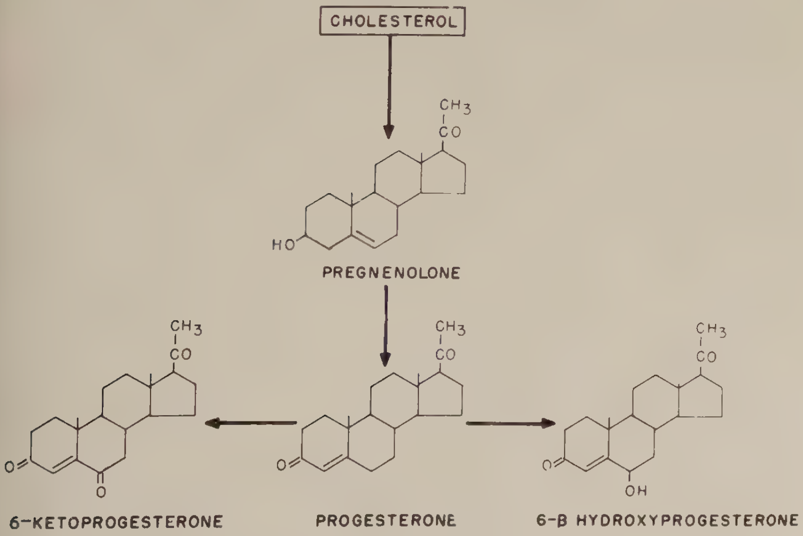

FIGURE 3

droxylation has been demonstrated in other organs such as the adrenal, the ovary, and the liver. It is likely that these substances actually are secreted by the placenta since their metabolites have been isolated from normal pregnancy urine.[27] Their biological significance is obscure. Both are weakly progestational substances.

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PLACENTAL TRANSFER OF THYROTROPIC HORMONE, THYROXINE, TRIIODOTHYRONINE, AND INSULIN IN THE RAT*

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Although an impressive body of knowledge relating to fetal endocrine function has been been amassed in recent years, the interrelationships and interactions between the maternal and fetal endocrine systems have received but scant attention. The influence of maternal hormones on fetal development in general, and on the fetal endocrine system in particular, is in large measure a matter for conjecture. The acquisition of understanding in this last area is largely dependent on information concerning the permeability of the placenta to maternal hormones.

This paper is concerned primarily with the placental transfer of hormones from mother to fetus in the rat as studied in our laboratory by the use of experimental approaches designed to provide indirect evidence for such transfers.

Thyrotropic Hormone

The administration of exogenous thyrotropic hormone (TSH) to pregnant animals [1-3] in attempts to stimulate the fetal thyroid gland has yielded negative results with the conclusion that TSH is, in all probability, incapable of crossing the placental barrier. The possibility remains, however, that endogenously secreted maternal TSH can do so and thus influence fetal thyroid function.

The following study‡ was initiated to determine the influence of increased and reduced quantities of circulating maternal TSH on the fetal thyroid gland and the role of maternal TSH in goiter formation in the fetus following the feeding of propylthiouracil (PTU) to the mother.

Methods. Pregnant rats of the Sprague-Dawley strain were hypophysectomized or thyroidectomized on the twelfth day of gestation.[5] Some of the hypophysectomized animals were given 0.1 per cent PTU in the diet starting on the day of operation. In addition, sham-operated pregnant rats were fed a 0.1 per cent PTU diet from the twelfth day of pregnancy. Intact sham-operated pregnant rats served as absolute controls. All animals were sacrificed on the twenty-first day of gestation; the fetuses were removed and weighed, and their thyroid glands (pooled glands from 4 fetuses made up one observation) weighed on a torsion balance. In two groups of pregnant rats, one hypophysectomized and the other intact, 20μc. of I^{131} was administered 24 hours prior to autopsy, and the radioactive iodine uptakes of the fetal thyroid glands were determined.

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‡ A preliminary report of this study was presented at The 1955 Meeting of The Endocrine Society, Atlantic City, N. J.[4]

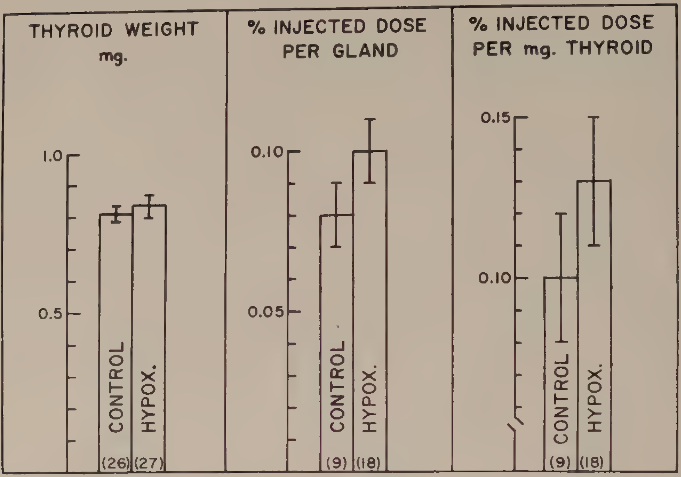

FIGURE 1. The effect of hypophysectomy of mothers, on the twelfth day of gestation, on fetal thyroid weight and fetal thyroid I^{131} uptake as measured on the twenty-first day of gestation. The vertical lines represent two standard errors; the figures in parentheses, the number of observations.

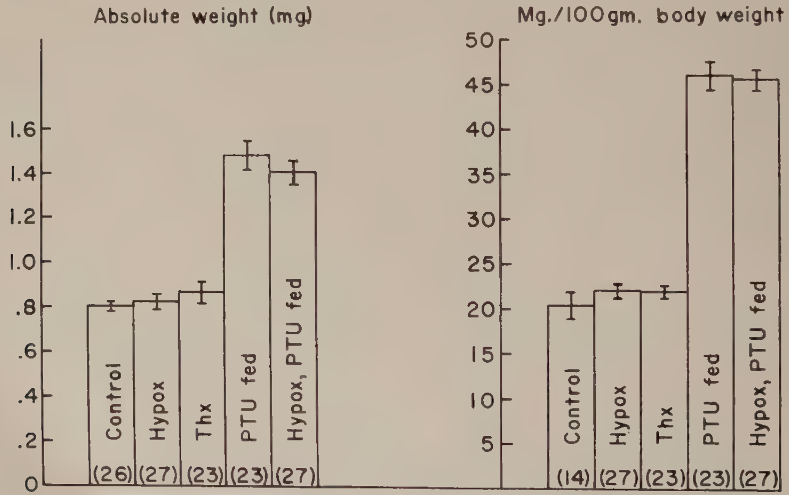

FIGURE 2. The influence of maternal thyroidectomy, hypophysectomy, and PTU feeding, on the twelfth day of gestation, on fetal thyroid weight on the twenty-first day of pregnancy.

Results. The reduction in circulating maternal TSH by hypophysectomy of the mother failed to influence fetal thyroid I^{131} uptake and fetal thyroid weight (FIGURES 1 and 2). Thyroidectomy of the mother, a procedure that increases circulating levels of maternal TSH, was also without effect on fetal thyroid weight (FIGURE 2). The administration of PTU to pregnant rats, however, effected a highly significant increase in fetal thyroid weight, which was not prevented or modified by prior hypophysectomy of the mother (FIGURE 2).

Conclusion. The above observations lead to the conclusion that: (1) goiter formation in the fetus following PTU feeding to pregnant rats is mediated by the fetal pituitary-thyroid axis, rather than by an increased secretion of maternal TSH; and (2) TSH of maternal hypophyseal origin does not influence the fetal thyroid gland, whether present in increased or decreased quantities, presumably because of its inability to cross the placental barrier.

Thyroid Hormones

The exchange of thyroid hormones between the maternal and fetal circulations, while the object of numerous studies using both direct and indirect criteria of placental transfer, has remained a matter of controversy. Courrier and Aron[6] concluded that thyroxine does not cross the placenta in the dog and the guinea pig. Peterson and Young,[3] on the other hand, were able to suppress fetal goitrogenesis in the guinea pig following the administration of PTU to the mother by the simultaneous injection of thyroxine. While strongly suggestive of placental transfer of this hormone, the role of the maternal pituitary was not ruled out (see previous section). More recent studies in the rabbit, the guinea pig, and man,[7-9] employing the recovery of labeled hormone in the fetal circulation after its injection to the mother and vice versa, have yielded results that support the conclusion that the placenta is largely impermeable to thyroxine and triiodothyronine. Postel[10] reports that triiodothyronine, when administered to pregnant guinea pigs, was totally unable to suppress fetal goitrogenesis induced by perchlorate; he concludes that the placenta in this species is impermeable to triiodothyronine.

The following study* was undertaken to determine the placental permeability to thyroxine and triiodothyronine in the rat.

Methods. The experimental design employed is based on the finding of the previous study that maternal TSH does not play a role in fetal goitrogenesis induced by PTU feeding to the mother. It follows, therefore, that the effect of PTU on the fetal thyroid is in all likelihood due to the hypersecretion of fetal TSH, and that the suppression of such goitrogenesis by thyroid hormones administered to the mother may be taken as evidence of the passage of these hormones to the fetal circulation. Pregnant rats were offered a diet containing 0.1 per cent PTU beginning with the eleventh day of gestation. Daily injections of thyroid hormones or saline were instituted at the same time. Pregnant rats maintained on a normal diet and receiving daily saline injections beginning with the eleventh day of pregnancy served as controls. In the initial experiment, L-thyroxine and L-triiodothyronine were administered in a dose of 15 μg. per day. In subsequent experiments these hormones were injected at dose levels ranging from 0.5×10^{-9} to 9.0×10^{-9} M/100 gm. body weight/day. All animals were sacrificed on the twenty-first day of gestation, one day following the last injection, and their thyroids weighed on a torsion balance. Fetuses and their thyroid glands were handled as described in the previous section. Pools of 3 to 4 fetal thyroid glands made up one observation.

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* A preliminary report of this study was presented at the 1957 meeting of The Endocrine Society.[11]

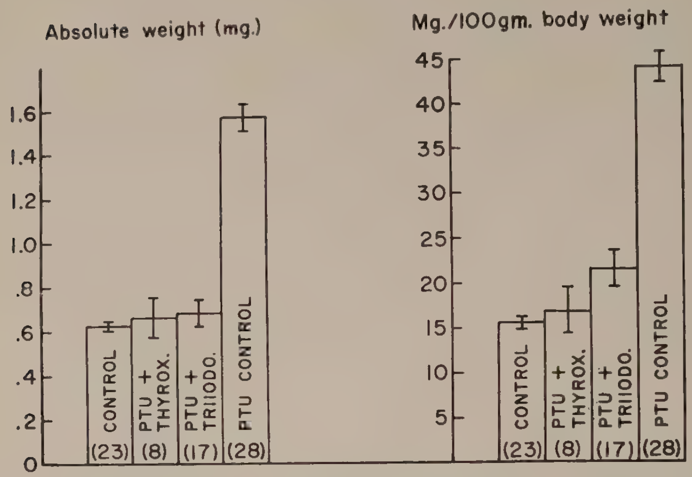

FIGURE 3. Inhibition of fetal goiter formation by thyroxine and triiodothyronine (15 μg./day) injected into pregnant rats receiving 0.1 per cent PTU.

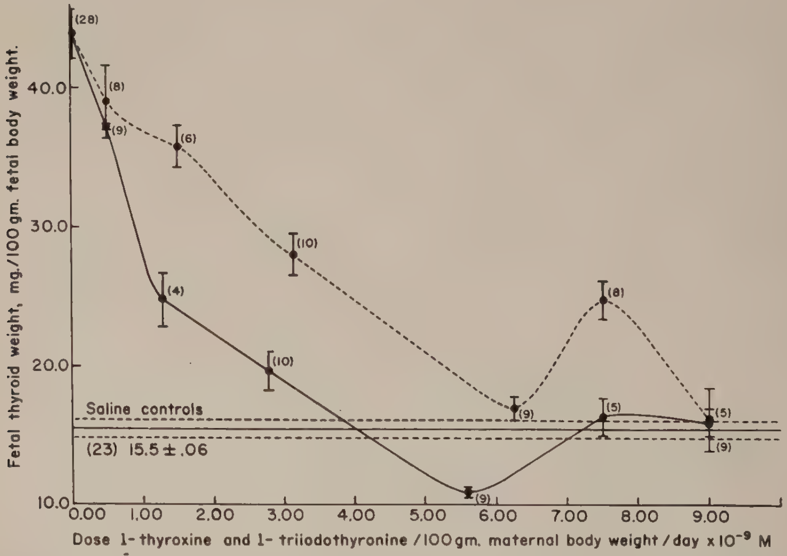

FIGURE 4. Relative effectiveness of thyroxine and triiodothyronine in suppressing fetal goiter formation in response to PTU feeding to the mothers. The solid line represents thyroxine; the dotted line, triiodothyronine.

Results. It is clear from FIGURE 3 that the administration of thyroxine or triiodothyronine in daily doses of 15 μg. abolished the increase in fetal thyroid weight resulting from feeding PTU to the mother. Surprisingly, thyroxine was approximately two times more effective than triiodothyronine in suppressing fetal goitrogenesis (FIGURE 4). In contrast, triiodothyronine was slightly

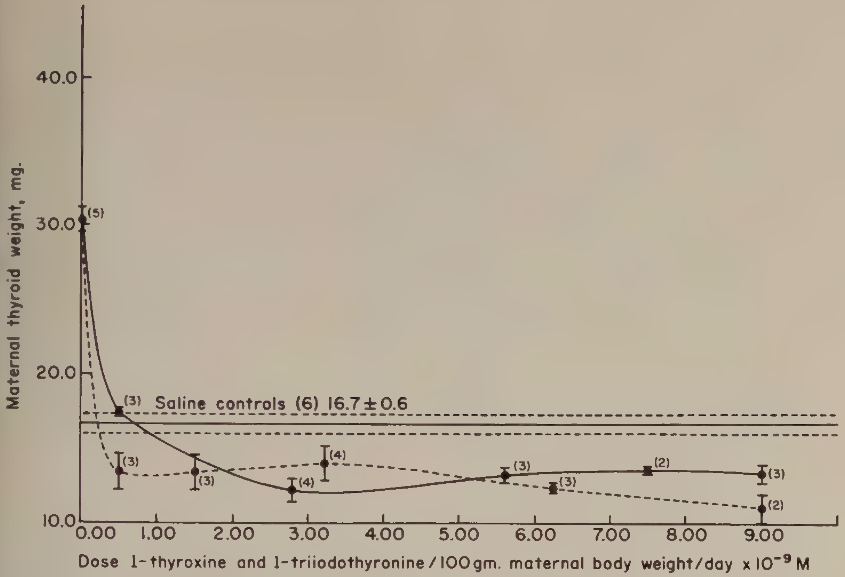

FIGURE 5. Relative effectiveness of thyroxine and triiodothyronine in suppressing maternal goiter formation in response to PTU feeding. The solid line represents thyroxine; the dotted line, triiodothyronine. Numbers in parentheses are numbers of pregnant animals.

more effective than thyroxine in inhibiting goiter formation in the mother (FIGURE 5).

Conclusion. It appears evident from the foregoing that, under the experimental conditions employed, both thyroxine and triiodothyronine can cross the placental barrier in the rat in quantities sufficient to inhibit the fetal pituitary gland's secretion of TSH. The unexpectedly greater effectiveness of thyroxine in this regard suggests that this hormone can traverse the placental barrier more readily than can triiodothyronine. This could be explained by differences in the placental binding of the two hormones.[12] The possibility remains that triiodothyronine was metabolized more rapidly than thyroxine, thus reducing the effective blood levels of triiodothyronine in the fetus. It was found in this study, however, that dividing the daily dose into two injections did not modify the results obtained. Since much larger doses of thyroxine and triiodothyronine are necessary to inhibit fetal goiter formation than are required to suppress maternal goitrogenesis, it would appear that the placenta limits the diffusion of these hormones from the maternal to the fetal circulation. Nevertheless, it seems clear that this partial barrier to the transfer of thyroid hormones from mother to fetus permits the access of physiologically effective amounts of these hormones to the fetus.

The statistically significant increase in fetal thyroid weight when the dose of hormones was increased to 7.5×10^{-9} M is a puzzling phenomenon that was confirmed by repeated experiments. A plausible explanation for it is not forthcoming at the present time.

Insulin

Previous attempts to investigate the permeability of the placenta to insulin have been few, and the results obtained have been inconclusive at best. For the most part, they have involved the administration of insulin to the fetus in the goat,[13] the bitch,[14, 15] and the cat.[15] These studies all yielded similar results, namely, that the injection of insulin into the fetus reduced the maternal blood glucose concentrations. Since the placenta appears to be freely permeable to glucose in all species studied,[16] the above observations made interpretation of the data difficult, since insulin-induced hypoglycemia in the fetus might well result in a secondary maternal hypoglycemia as a result of an equilibration of the sugar concentrations between fetal and maternal bloods.

This study* was undertaken to re-examine the problem of the passage of insulin across the placenta by use of a method that excludes equilibration of glucose concentration between the maternal and fetal circulations as an uncontrolled variable.

Methods. Preliminary experiments were performed to determine the minimal dose of insulin which, when administered intravenously to rats anesthetized with sodium pentobarbital, would effect a minimal reduction in the blood sugar concentrations. This dose was found to be 0.10 units crystalline insulin dissolved in physiological saline.

Pregnant rats were subjected to a 2- to 6-hour fast on the twenty-first day of gestation, anesthetized with sodium pentobarbital, and a midline laparotomy performed. A ligature was placed around the cervical end of the right uterine horn, which included the continuation of the right superior vesical artery after the cranial branching of the uterine artery (FIGURE 6). Under a dissecting microscope and with iris forceps the uterine artery was freed from its connective tissue sheath near the ligature. Topical application of 5 per cent procaine hydrochloride in saline reduced arterial spasm. The exposed artery was placed under tension and injected with a tuberculin syringe equipped with a 30-gauge needle. Intra-arterial injections of 0.1 ml. saline or 0.05 units of insulin in 0.1 ml. of saline were achieved in this manner. Maternal and fetal blood samples for the determination of blood sugar concentration were obtained in the following sequence. Before injection, one fetus was removed from the ovarian end of *each* uterine horn through an incision in the uterine wall. The chest of the fetuses was incised and the heart was cut with iridectomy scissors. A 0.1 ml. sample was withdrawn from the blood accumulated in the chest cavity. A 0.1 ml. sample of maternal blood was taken immediately thereafter from the tail vein. This sequence of sampling of fetal and maternal blood was repeated at approximately 10-minute intervals following the injection of insulin or saline into the right uterine artery. The various blood samples were analyzed for reducing sugar content by the method of Nelson.[18]

The effect of insulin on newborn rats was determined by the intravenous injection of 0.05 units of crystalline insulin in 0.05 ml. of saline to unanesthetized rats between 1 and 2 days old. Control neonatal rats were given equal

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* A preliminary report of this study was presented at the 1957 meeting of The Endocrine Society.[17]

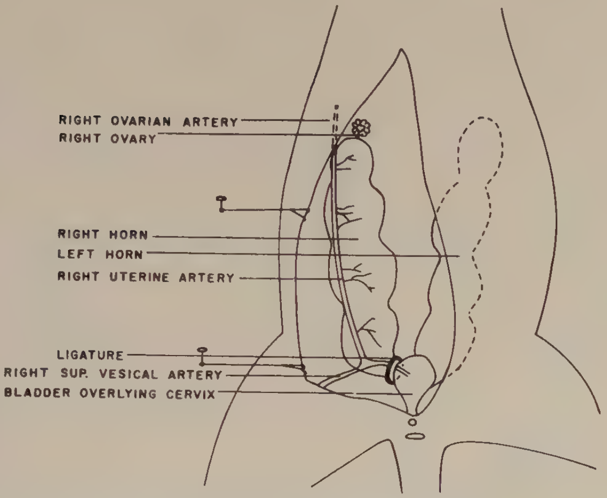

FIGURE 6. Schematic diagram of the surgical field for the injection of insulin or saline into the right uterine artery of the pregnant rat.

volumes of physiological saline. Injections were made in the tail vein under a dissecting microscope. Blood samples were obtained as above.

Results. The injection of 0.9 per cent saline into the right uterine artery resulted in a rather consistent elevation in the blood sugar concentrations observed in the fetuses from the injected horn when compared to the blood sugar concentrations observed in the fetus from the uninjected horn or to the maternal blood sugar concentration. A significant reduction in the blood sugar concentration in the injected horn fetuses was never observed when saline alone was administered.

FIGURE 7 illustrates one of the thirteen experiments in which insulin was injected into the uterine artery of the right uterine horn. It shows that the maternal blood sugar concentration rose with time, as did the blood sugar concentration of the fetuses removed from the uninjected horn. In contrast, a marked reduction in blood sugar concentration was noted in the fetuses removed from the injected side forty five minutes after the intra-arterial administration of insulin. Since there was considerable variation in maternal and fetal blood sugar concentrations from experiment to experiment, the results were normalized in the manner indicated in FIGURES 8 and 9 to permit pooling of the data and statistical evaluation. The time course of blood sugar concentrations in fetuses removed sequentially from the insulin-injected side and noninjected side relative to the maternal blood sugar concentration is illustrated in FIGURE 8. It is clear that the blood sugar concentrations of fetuses in the injected horn are significantly lower than those removed at the same time from the control horn and are also significantly lower than the maternal blood sugar concentration. The data are perhaps more striking when the blood sugar concentrations of fetuses from control and injected horns are compared at the time of maximal

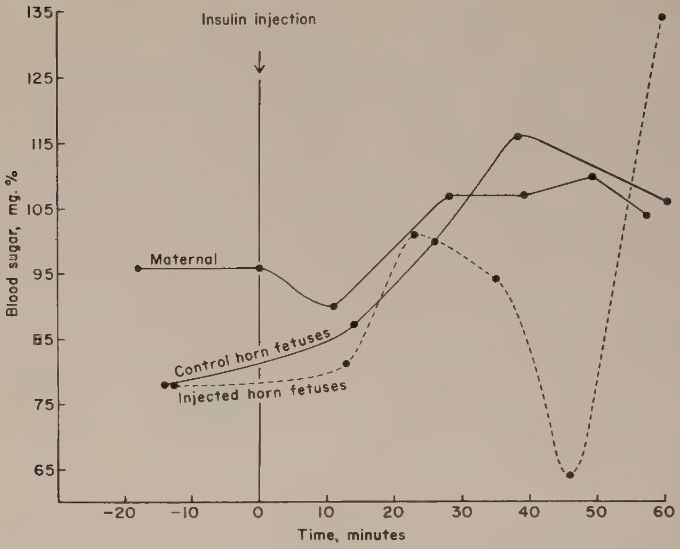

FIGURE 7. Time course of fetal and maternal blood sugar concentrations in one rat before and after the injection of 0.05 units of crystalline insulin into the uterine artery of the right uterine horn.

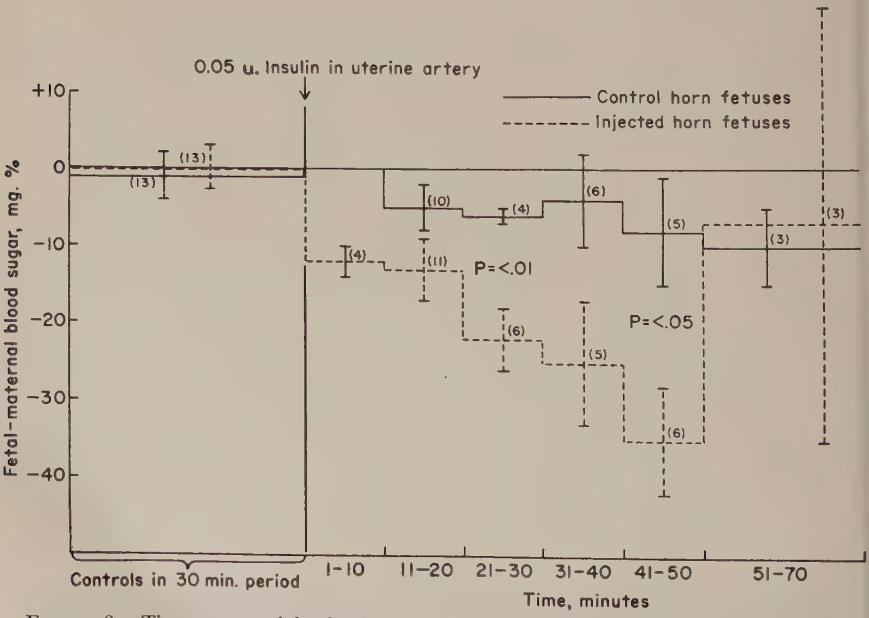

FIGURE 8. Time course of fetal minus maternal blood sugar concentrations in thirteen experiments before and after the injection of 0.05 units crystalline insulin into the right uterine artery.

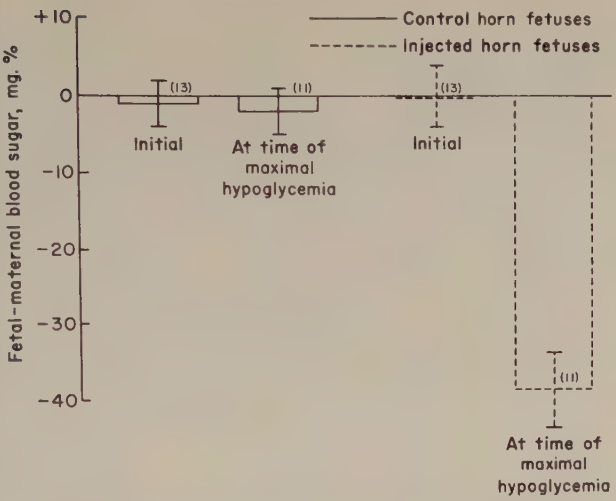

FIGURE 9. Difference between fetal blood sugar concentrations relative to maternal blood sugar concentration before the injection of insulin and at the time that fetal hypoglycemia was maximal in each horn.

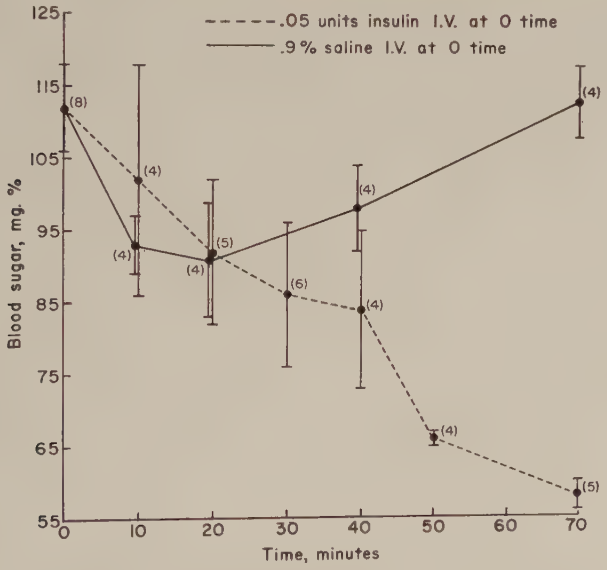

FIGURE 10. Effect of the intravenous injection of insulin and saline into newborn rats on blood sugar concentrations.

hypoglycemia in each horn (FIGURE 9). The time course of insulin-induced hypoglycemia in newborn rats is quite comparable to that observed in fetuses where the placenta was interposed between the site of insulin injection and the fetal circulation (FIGURE 10).

Conclusion. The foregoing results appear to be subject to but one interpretation: the insulin injected directly into the uterine artery traversed the placenta and exerted a hypoglycemic action in the fetus. To our knowledge this is the first conclusive demonstration of the placental transfer of insulin from the maternal to the fetal circulation.

Summary

(1) Evidence is presented suggesting that, in the rat, maternal TSH does not cross the placental barrier, and that goiter formation in the fetus consequent to propylthiouracil feeding to the mother is due entirely to an increased secretion of fetal TSH, since it occurs in the absence of the maternal pituitary gland.

(2) Thyroxine and triiodothyronine, when injected into pregnant rats, are capable of suppressing fetal TSH secretion, presumably by being transferred across the placenta from the maternal to the fetal circulation. Thyroxine is approximately 100 per cent more effective than triiodothyronine in this regard, and the inference is made that the placenta in the rat may be more permeable to to thyroxine than to triiodothyronine. A functional barrier, however, limits the entrance of both hormones into the fetal circulation.

(3) Experiments are described that conclusively demonstrate that in the rat insulin is able to cross the placental barrier from the maternal to the fetal circulation and to exert a hypoglycemic action in the fetus.

Acknowledgments

We are indebted to A. E. Hemming of the Smith, Kline and French Co., Philadelphia, Pa., for supplies of L-thyroxine and L-triiodothyronine.

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CHROMOSOMAL SEX DETECTION IN THE HUMAN NEWBORN AND FETUS FROM EXAMINATION OF THE UMBILICAL CORD, PLACENTAL TISSUE, AND FETAL MEMBRANES*

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Five years ago Barr and his associates (Moore *et al.*, 1953) announced a new and relatively simple histological method utilizing a skin biopsy for the diagnosis of sex. This is based upon the fact that intermitotic nuclei of females contain a characteristic mass of chromatin, the sex chromatin which is rarely, if ever, present in males. Since these particular chromocenters presumably represent fused heterochromatic portions of the XX sex-chromosomal complex of females, nuclei possessing the sex-chromatin body are found only in specimens from chromosomal females (Barr, 1957; Reitalu, 1957).

Rapidly confirmed by numerous investigators (Hunter *et al.*, 1954; Marberger and Nelson, 1954; Emery and McMillan, 1954; Sohval *et al.*, 1955), these observations have been applied to a variety of anatomical preparations and clinical conditons. As a result there have been two salient developments: first, cytological methods for the detection of chromosomal sex have been described employing buccal scrapings (Moore and Barr, 1955; Marberger *et al.*, 1955), smears from the vagina and urogenital sinus (Carpentier *et al.*, 1956), leukocytes from the peripheral blood (Davidson and Smith, 1954), and cells of the amniotic fluid obtained prenatally (Serr *et al.*, 1955; James, 1956). In the last instance, the cells are of fetal origin, derived principally from the mucosae of the alimentary and genitourinary tracts rather than from the skin or amnion (Rosa and Fanard, 1951; Lennox, 1956; Makowski *et al.*, 1956). In addition, a nuclear sex difference has been observed histologically by Marberger and Nelson (1955) in mesenchymal cells of the amnion of human fetal membranes, by Roitman (1955) in the nuclei of smooth muscle cells of the umbilical vessels, by Glenister (1956) and by Park (1957) in early human trophoblastic nuclei, and by Bohle and Hienz (1956) in the connective tissue and endothelial cells of human term placental villi. Second, the determination of chromosomal sex in certain patients with congenital errors of sex development has contributed significantly to our understanding of the pathogenesis and to the management of these conditions.

The purpose of this communication is to report the results of a systematic investigation of the nuclear sex chromatin in the umbilical cord, placental tissue, and fetal membranes of the human newborn and of fetuses of various ages. The demonstration of concordant nuclear sex differences in these structures represents an additional technique for chromosomal sex detection. Some advantage accrues from the fact that availability or manipulation of the newborn or fetus itself is not required.

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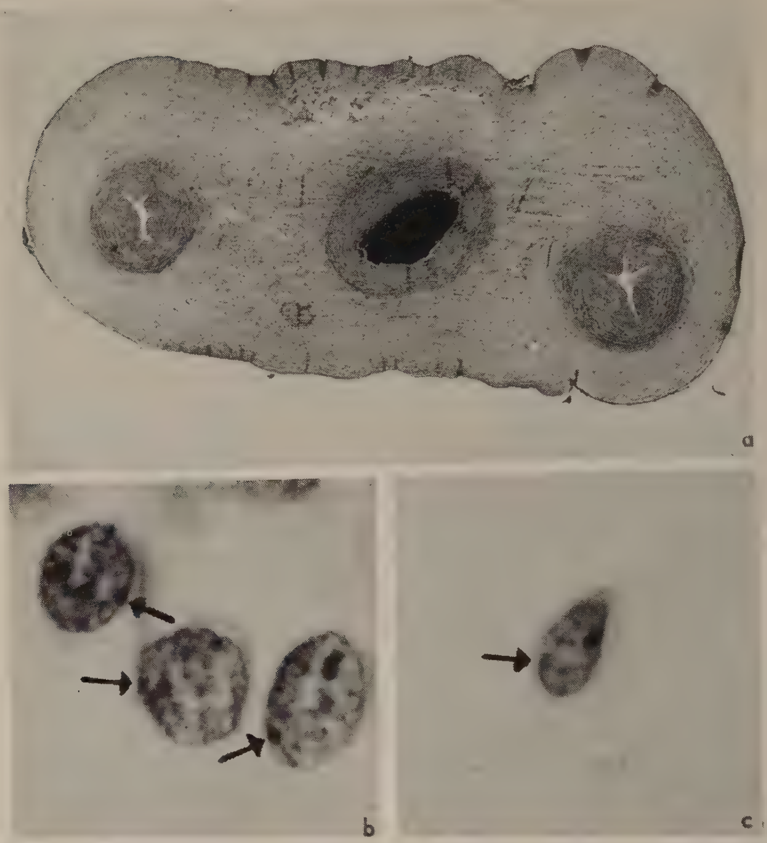

FIGURE 1. Umbilical cord associated with female newborn infant. (a) Low-power view (H.& E., ×10). (b) Sex chromatin mass (arrows) in the nucleus of each of three epithelial cells lining the umbilical cord (H.& E. ×1600). (c) Sex chromatin (arrow) in the cell nucleus of Wharton's jelly (H.& E., ×1600).

Material and Method of Study

Fresh specimens were obtained from the umbilical cords and placentas of 105 apparently normal newborn infants, 55 of which were male and 50 were female. In each instance a segment of the cord (FIGURE 1) 0.5 cm. in length and a portion of the placenta (FIGURE 2) measuring approximately 2.5 × 1.5 × 0.5 cm. were procured. The placental specimens were removed from marginal areas so that attached portions of the fetal membranes were included. After fixation in modified Davidson's solution (alcohol-formalin-acetic acid) for 24 hours, the tissues were immersed in repeated changes of 70 per cent alcohol for approximately 2 days and subsequently dehydrated and embedded in paraffin. Sections were cut at 5 microns and stained with hematoxylin and and eosin. A number of sections were also stained by the Feulgen method.

For comparative purposes an additional series of histological preparations

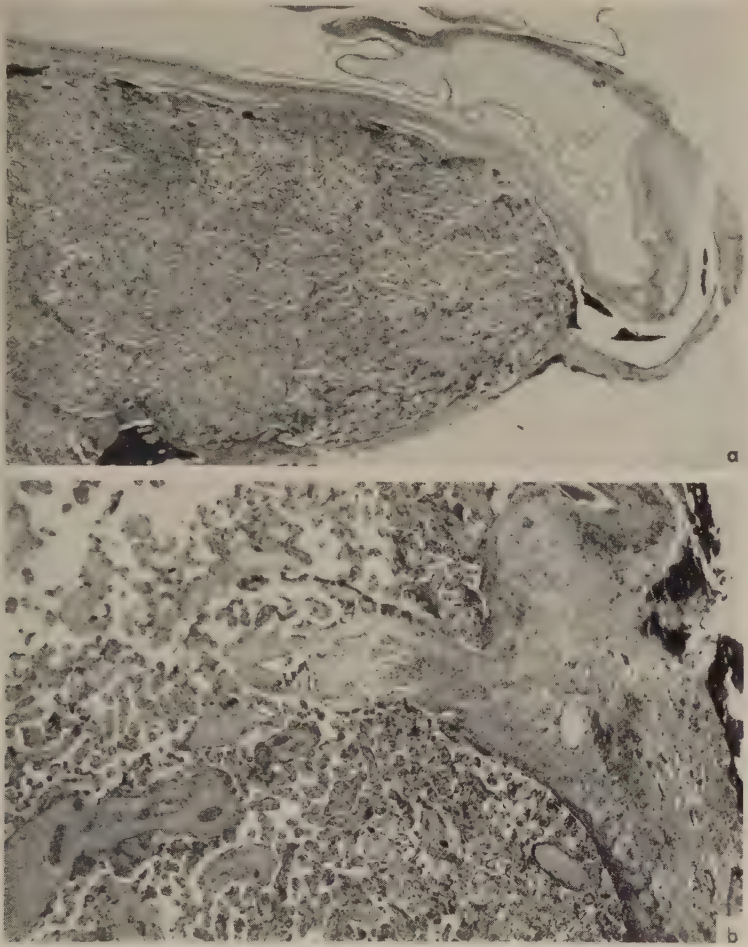

FIGURE 2. (a) Placental margin, showing villi enclosed between chorionic and basal plates. A portion of the amniotic sac is attached (H.& E., ×5). (b) Low-power view of the placental septum extending into the substance of the placenta from the basal plate (H.& E., ×25).

of placental tissue and umbilical cords of fetuses was examined. Procured at random from our surgical pathology files, these routine sections had been fixed in 10 per cent neutral formalin and stained with hematoxylin and eosin. The material was derived from the curettages of 19 therapeutic abortions (fetuses 6 to 10 weeks old) and from 28 instances of premature labor, the fetal ages ranging between 15 and 30 weeks. Each of the latter group had died intrapartum with the exception of one who succumbed a few hours postnatally. Premature separation of the placenta was the cause of premature labor in the majority of cases. Only one of the fetuses was known to be abnormal, showing calcification of the large vessels. Of the 28 prematurely delivered fetuses, 17 were described grossly as female and 8 as male. The sex was not established in 2 fetuses 16 weeks of age, and in another 17 weeks old.

Microscopic examination was performed under oil immersion routinely at a magnification of 1250 diameters and, occasionally, at 2000. Knowledge of the genital sex of the newborn or of the fetus was not available to the observers at the time of nuclear sexing.

Ordinarily, identification of the sex-chromatin body is optimally accomplished in cells with vesicular nuclei having fine chromatin detail. This conspicuous chromocenter is approximately one micron in diameter and it characteristically lies against the nuclear membrane. Occasionally, it is seen free in the nucleoplasm, but it is recognized most easily when it lies at the periphery of the optical section through the nucleus (Barr, 1955). The sex chromatin is generally considerably larger than other particulate chromatin material and is therefore readily distinguished from those chromatin particles that may be in contact with the nuclear membrane by chance. While usually planoconvex in shape, its inner aspect bulging centrally, the sex chromatin may also assume a disc-shaped, triangular, circular, bell-shaped (Moore and Barr, 1954) and even flattened outline (Moore and Barr, 1955). In the last instance it may appear as a localized thickening of the nuclear membrane. Under certain conditions of optical resolution a minute central area of pallor can be discerned The sex chromatin is stained intensely with hematoxylin. Because of its rich content of desoxyribose nucleic acid, it is Feulgen-positive (in contrast to nucleoli that contain ribose nucleic acid and may also be peripherally situated).

The reported incidences of sex chromatin in the nuclei of females vary from approximately 50 to 80 per cent in the epidermis (Barr, 1957; Nelson, 1956) to 30 to 60 per cent in oral smears (Barr, 1957; Nelson, 1956) and in cells obtained from amniotic fluid (Makowski *et al.*, 1956; Shettles, 1956). In the present study, no attempt was made to compute the exact percentile range of nuclei containing the sex chromatin. Accurate calculations were difficult and deemed impracticable because of the large numbers of nuclei that were unsuitable for interpretation. For example, many chorionic nuclei contained coarsely granular and abundant amounts of chromatin particles that obscured or even simulated sex chromatin. In addition, numerous different nuclei showed pyknosis and other degenerative changes. For these reasons, rather rigid criteria for identification, employing well-preserved nuclei of good technical quality with delicate chromatin structure, were maintained. Moreover, a chromocenter was not regarded as sex chromatin unless its basal surface was in close contact with the nuclear membrane. Thus, prolonged search was occasionally necessary to find readable cells in which one could be reasonably certain that sex chromatin would be identified if it were present. Under these circumstances, it was felt that the expression of an estimated incidence, based upon the impressions of the observers, would be more informative.

Results

Wherever present, the anatomic components of the umbilical cord, placental tissue, and fetal membranes were subjected to systematic study (TABLE 1).

TABLE 1

ESTIMATED PERCENTAGE INCIDENCE OF SEX CHROMATIN IN NUCLEI OF EXTRAFETAL PRODUCTS OF CONCEPTION

	Female fetus	Male fetus
Umbilical Cord:		
Lining epithelial cells	30–50	0
Cells of Wharton's jelly	30–50	0
Placental Tissue:		
Epithelial cells on fetal surface	10–20	0
Subamniotic connective tissue cells	20–50	0
Perivascular connective tissue cells	20–50	0
Chorionic cells embedded in chorionic plate	10–20	0
Chorionic villi:		
Stromal cells	10–20	0
Cytotrophoblastic cells	10–20	0
Syncytiotrophoblastic cells	—	—
Special "trophoblastlike" cells*	10–20	10–20
Decidual cells	10–30	10–30
Fetal Membranes:		
Amnion:		
Lining epithelial cells	10–20	0
Connective tissue cells	20–50	0
Chorionic cells	10–20	0
Decidual cells (fortuitously attached)	10–30	10–30

* Referred to in the text as cells of ill-defined origin, situated predominantly in the basal plate and placental septa, where they are embedded in a fibrinoid matrix.

While formalin-fixed preparations were often suitable for analysis, nuclei of good technical quality were encountered more frequently in specimens which had been fixed in Davidson's solution. To the trained observer, the Feulgen technique offered little or no advantage over the hematoxylin-eosin method of staining.

Umbilical cord. The cell nuclei of Wharton's jelly were particularly suitable for interpretation of nuclear sex while the nuclei of the layer of cuboidal or flattened epithelial cells enclosing the cord were readable approximately one half as often. The least useful were the endothelial and muscle nuclei of the umbilical arteries and veins.

Sex chromatin was identified in approximately 30 to 50 per cent of the readable nuclei of the umbilical cords from the 50 full-term female infants (FIGURE 1). Definite sex-chromatin masses were not encountered in any of the specimens obtained from the 55 males in this group.

Specimens of umbilical cord were available in 15 of the 28 cases of premature labor. In general, these formalin-fixed preparations possessed fewer nuclei of good technical quality than those of the larger group that had been fixed in Davidson's solution. Nevertheless, an interpretation of nuclear sex could be rendered in 14 of the 15. The findings of sex chromatin in 30 to 50 per cent of the nuclei of 7 specimens and in none of the 7 others was in complete agreement with the sex of the fetus as determined by examination of the genitalia.

Placental tissue. The different cellular constituents of the placenta displayed varying readability of their nuclei. The nuclei of young connective tissue cells were most satisfactory, principally because of their comparativ freedom from degenerative changes. They were abundantly present in perivascular connective tissue situated within the substance of the placenta an in the subamniotic connective tissue layer on the fetal aspect. The nuclei leas satisfactory for identification of sex chromatin were those of the syncytiotrophoblast. In fact, excessive and coarse chromatin granulation made it virtuall impossible to ascertain the presence or absence of sex chromatin in these nuclei On the other hand, cytotrophoblastic nuclei adequate for interpretation (FIGURE 5) were encountered fairly frequently in specimens obtained during the first and second trimesters of pregnancy. Quite satisfactory were villa stromal nuclei and nuclei of fibrinoid-embedded cells in the chorionic plate Decidual nuclei in the basal plate of the placenta, comprising the materna component of the structure, were usually readable despite the frequency o autolytic and other degenerative changes. In some preparations, the nucle of certain unit structures such as the stromal cells of the villi or the fibrinoid-embedded cells in the chorionic plate were of inferior technical quality becaus of shrinkage or degeneration, and they were therefore unsuitable for identification of sex chromatin. Because of the variations in readability of the different placental components, it was held essential to examine each unit separately and systematically. In this manner the superior quality of certain types of nuclei compensated for the shortcomings of others, so that the presence or absence o the distinctive sex-chromatin mass could be established for the tissue as a whole.

Decidual nuclei were frequently present in placental specimens and meticulous care was exercised to exclude them from interpretation because of their maternal origin. Nevertheless, they were useful controls, particularly when sex chromatin could not be identified in other placental nuclei.

In the group of 105 newborn infants, the fetal nuclei of 50 placentas contained sex chromatin sufficiently often to warrant the conclusion that these placentas originated from female embryos. This was confirmed in each instance by reference to the clinical records. The frequency of occurrence of the characteristic sex-chromatin mass in various placental constituents of good technical quality was approximately estimated as follows: 20 to 50 per cent of young connective tissue nuclei and 10 to 20 per cent of chorionic and villar stromal nuclei (FIGURES 3 and 4). Sex chromatin was not found in the fetal placental nuclei of the remaining 55 specimens, although on rare occasions these nuclei contained a chromatin structure stimulating sex chromatin. This was noted primarily in nuclei having coarse chromatin granulation that disqualified them from interpretation. Accordingly, each of these sections was regarded as of chromosomal male origin In each instance the diagnosis of male sex was substantiated by the clinical record.

Of the 28 placentas associated with spontaneous abortion or premature labor and fetal death, the nuclei of all but one were technically adequate for interpretation of nuclear sex. As was noted with the umbilical cords of this group, placental nuclei of good technical quality were fewer in these formalin-fixed specimens than in those fixed in Davidson's solution. This deficiency was in part compensated for by the presence of cytotrophoblastic nuclei that normally had not yet disappeared at the stage of pregnancy represented in this group. These

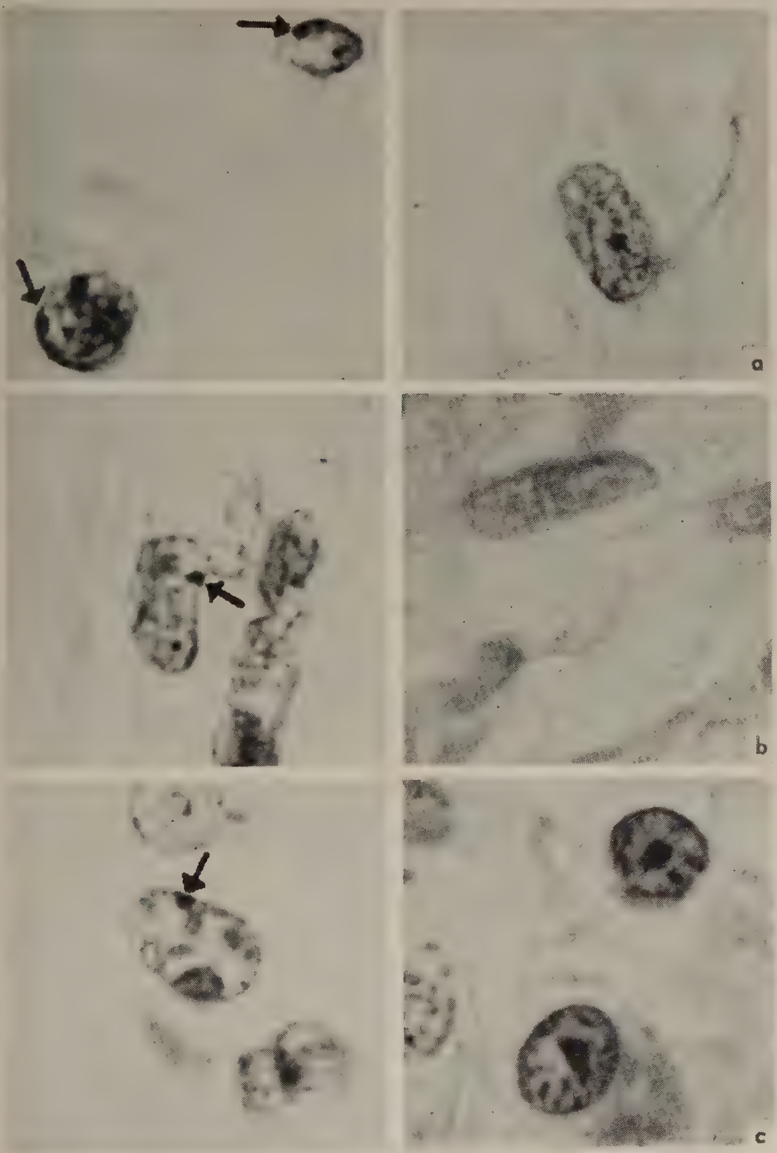

FIGURE 3. Placental nuclei from female (left) and male (right) newborn infants. The sex chromatin is indicated by arrows. (H. & E., ×1600). (a) Nuclei in the connective tissue layer beneath the fetal surface. (b) Nuclei of perivascular connective tissue. (c) Nuclei of chorionic cells embedded in the fibrinoid substance of the chorionic plate.

nuclei were readable (FIGURE 5) approximately one half as often as were other nuclei in the same preparations. Chromosomal females were diagnosed in 17 instances (TABLE 2) where the placental nuclei contained sex chromatin with a frequency similar to that noted in the preceding larger group. Characteristic

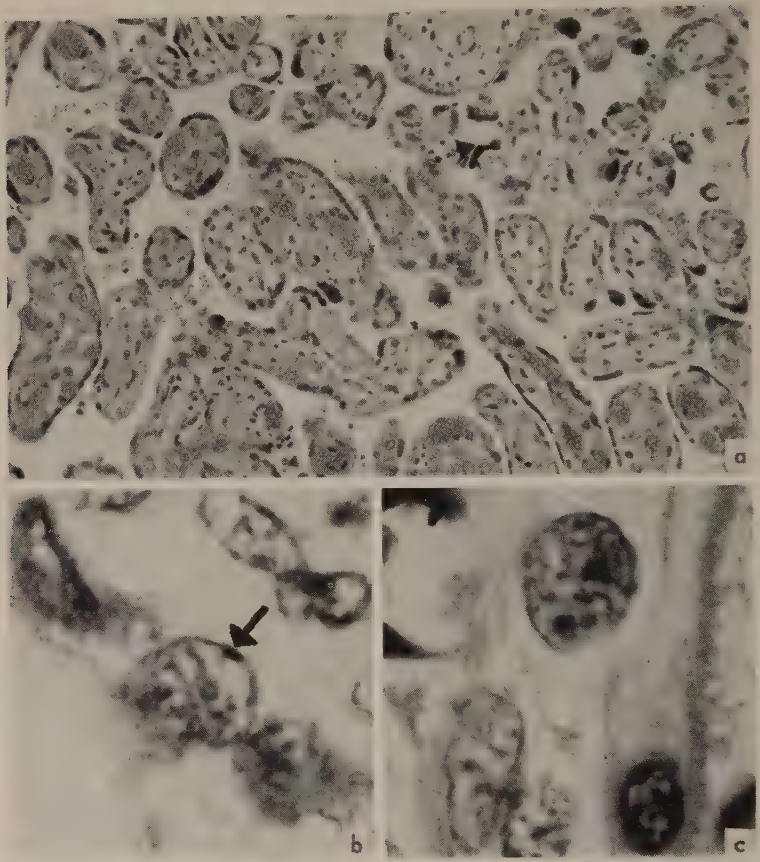

FIGURE 4. Term placental villi. (a) Low-power view showing the stromal and endothelial cells enclosed by syncytiotrophoblast (H.& E., ×100). Compare with FIGURE 5. (b) Sex chromatin (arrow) in a stromal nucleus in the placental villus of a female infant. It is not seen in corresponding nuclei (c) of villi associated with male newborns (H.& E., ×1600).

sex chromatin was not encountered in 10 cases that were accordingly interpreted as chromosomal males. These findings were in agreement with the genital sex, which was available in 25 fetuses. The genital sex of 2 fetuses 16 weeks of age was not known; the placental nuclei of one were male and the other, female. The placental nuclei of the third fetus of unknown genital sex, 17 weeks of age, contained no sex chromatin and were accordingly regarded as of male chromosomal sex. The single placenta in which the nuclei were technically inadequate originated from a female fetus.

The routine formalin-fixed sections of uterine curettings from 19 therapeutic abortions were the least satisfactory for identification of sex chromatin. Adequate technical quality was present in the stromal nuclei of the chorionic villi in 9 instances, in the cytotrophoblastic nuclei in 4 of these, and in the syncytiotrophoblastic nuclei in none. Sex chromatin was identified in approximately 10 to 20 per cent of villar stromal nuclei (FIGURE 5) in 5 preparations and in

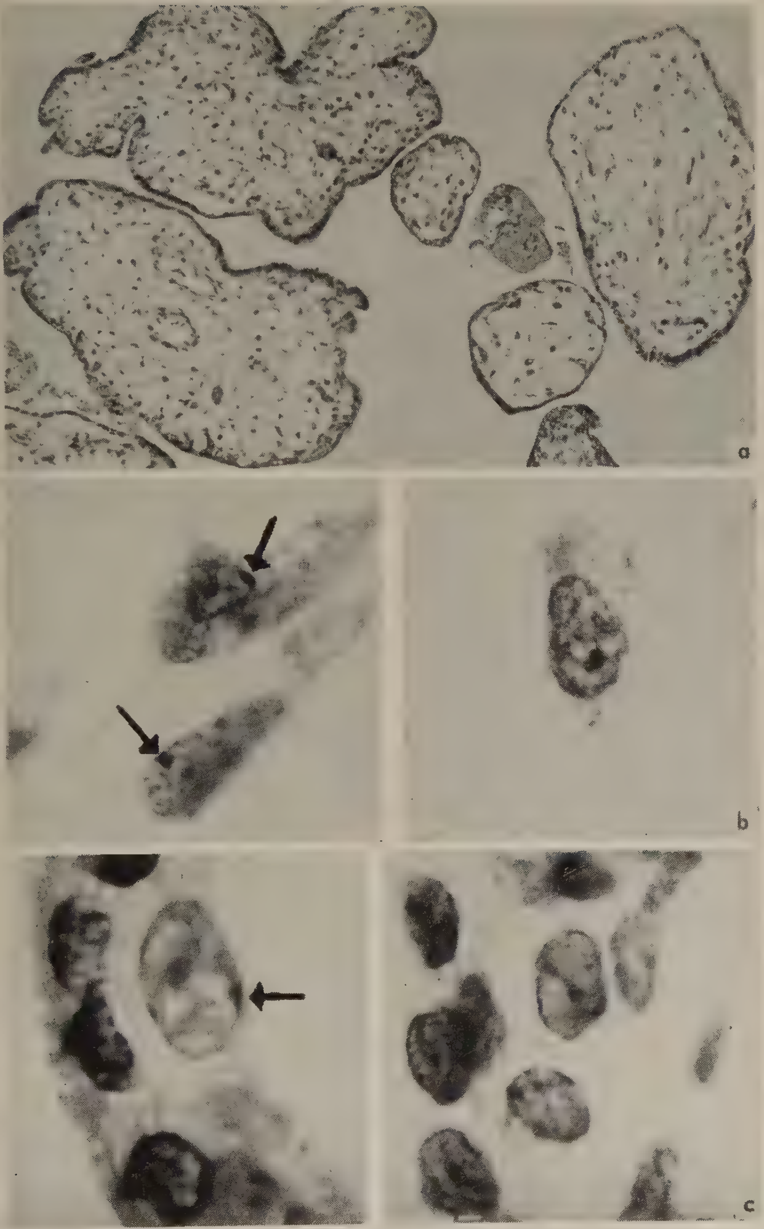

Figure 5. Chorionic villi in uterine curettings obtained during the eighth week of pregnancy. (a) Low-power view showing stromal and endothelial cells enclosed by a cytotrophoblast and syncytiotrophoblast (H.& E., ×100). The villi are larger and the stroma looser than at term. Compare with figure 4. (b) The villar stromal nuclei on the left contain sex chromatin (arrow) indicating that the embryo is a chromosomal female. The absence of sex chromatin from corresponding nuclei on the right indicates a male embryo (H.& E. ×1600). (c) Trophoblastic nuclei. A sex chromatin mass (arrow) is present in a cytotrophoblastic nucleus on the left, while none is seen in corresponding nuclei on the right, indicating a female and male embryo, respectively. The smaller, darker nuclei are syncytiotrophoblastic and cannot be interpreted because of their dense, coarse chromatin granulation (H.& E., ×1600).

Table 2

Comparison of Genital with Nuclear Sex in 28 Placentas Aged 15 to 30 Weeks

Nuclear sex	Genital sex		
	Male	Female	Unknown
17 Females		16	1
10 Males	8		2
1 Unknown		1	

none of the remaining 4. Thus, in 9 very early pregnancies in which the se was undifferentiated or undetermined, it was possible to recognize that 5 wer chromosomal females and 4 were males.

Whenever decidual cells were present, the estimated incidence of sex chromatin in their readable nuclei was 10 to 30 per cent, regardless of the stage o gestation.

Consideration of a particular type of placental cell has been reserved fo discussion at this point because of some uncertainity regarding its origin. We refer to aggregates of cells of ill-defined origin situated in the decidual plate and often extending into the placental septa toward the fetal surface (FIGURE 6) Less often, accumulations of similar cells are also found in placentas of variou ages as fairly large, more or less discrete nodules within the substance of the placenta in the intervillous spaces. The cells are always characteristically embedded in a homogeneous eosinophilic matrix usually referred to as fibrinoid The cytoplasm tends to be deeply basophilic. The round or ovoid nuclei are quite vesicular and are apt to contain increased amounts of coarsely granula chromatin material. When the cells lie adjacent to decidual cells, a certai resemblance between the two is apparent. However, these cells are somewhat smaller, their cytoplasm takes a more basophilic stain, and their nuclei are richer in chromatin. When localized collections of these cells are found dee in the placental mass in intervillous spaces they bear a close resemblance to chorionic cells embedded in the fibrinoid material of the chorionic plate.

The groups of cells in the decidual plate and placental septa appear to correspond to those described by Wislocki as constituting a persistent trophoblastic shell and, more recently by Latta and Beber (1957), as a special form o trophoblast. Employing various histochemical techniques, these investigators concluded that the cells are of fetal origin and that they probably account fo the continuing elaboration of chorionic gonadotropin after the disappearance o gonadotropin-producing typical cytotrophoblast at mid-pregnancy.

In the present investigation the nuclei of these cells were included in the study of sex chromatin. Although their chromatin content tended to be abundant with a proclivity toward clumping at the nuclear membrane, adequate numbers of suitable nuclei containing a delicate chromatin network were usually present. Since these special cells bear a certain resemblance to trophoblastic cells, they may be tentatively referred to as "trophoblastlike" cells.

Groups of special trophoblastlike cells were present in the placentas of 44

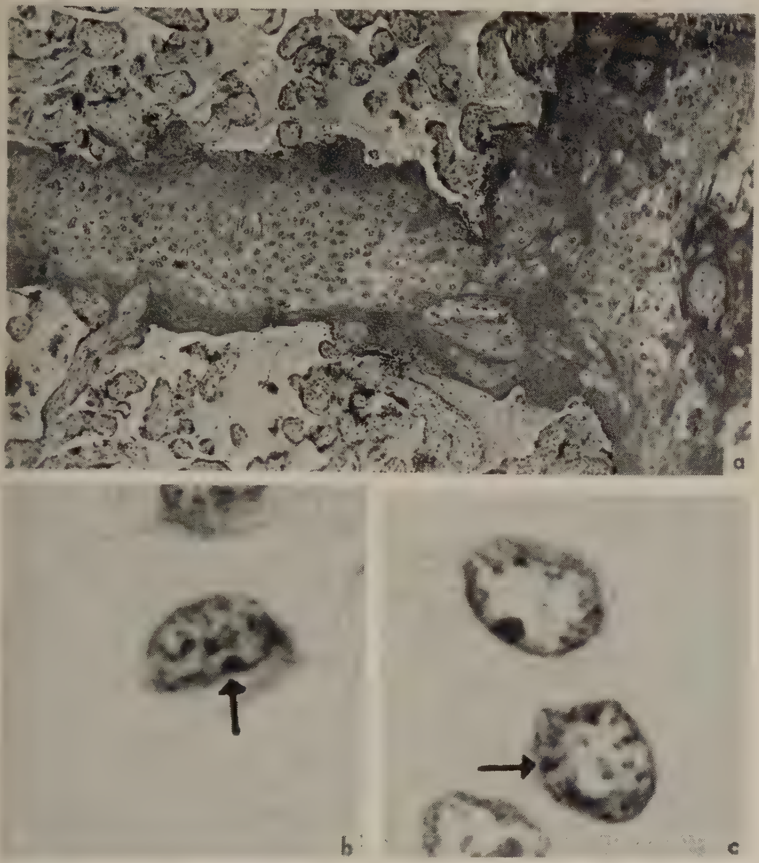

FIGURE 6. Special "trophoblastlike" cells. (a) Low-power view of a basal plate and lacental septum containing aggregates of cells with deeply basophilic cytoplasm embedded in n eosinophilic, homogeneous, fibrinoid matrix (H.& E., ×65). (b) A typical sex-chromatin nass (arrow) in a special trophoblastlike cell in septum of the placenta from a male newborn nd (c) from a female newborn (H.& E., ×1600).

ull-term male infants. Poor technical quality precluded nuclear sexing in 5 pecimens. A characteristic sex-chromatin mass was identified in 10 to 20 per ent of the readable nuclei of 37 sections (FIGURE 6). The presence of sex hromatin could not be ascertained in the remaining 2 cases, which contained elatively sparse numbers of the cells in question. The placentas of 46 full-erm female infants contained similar collections of special trophoblastlike ells. In 2 of these the nuclei were not suitable for interpretation. In the emaining 44 specimens with nuclei of good technical quality, sex chromatin was dentified in 10 to 20 per cent of the nuclei of 41. Its presence could not be stablished in 3 other sections with a paucity of readable nuclei.

Similar accumulations of special trophoblastlike cells were encountered in he placentas of 9 male and 15 female fetuses aged 16 to 30 weeks. As men-

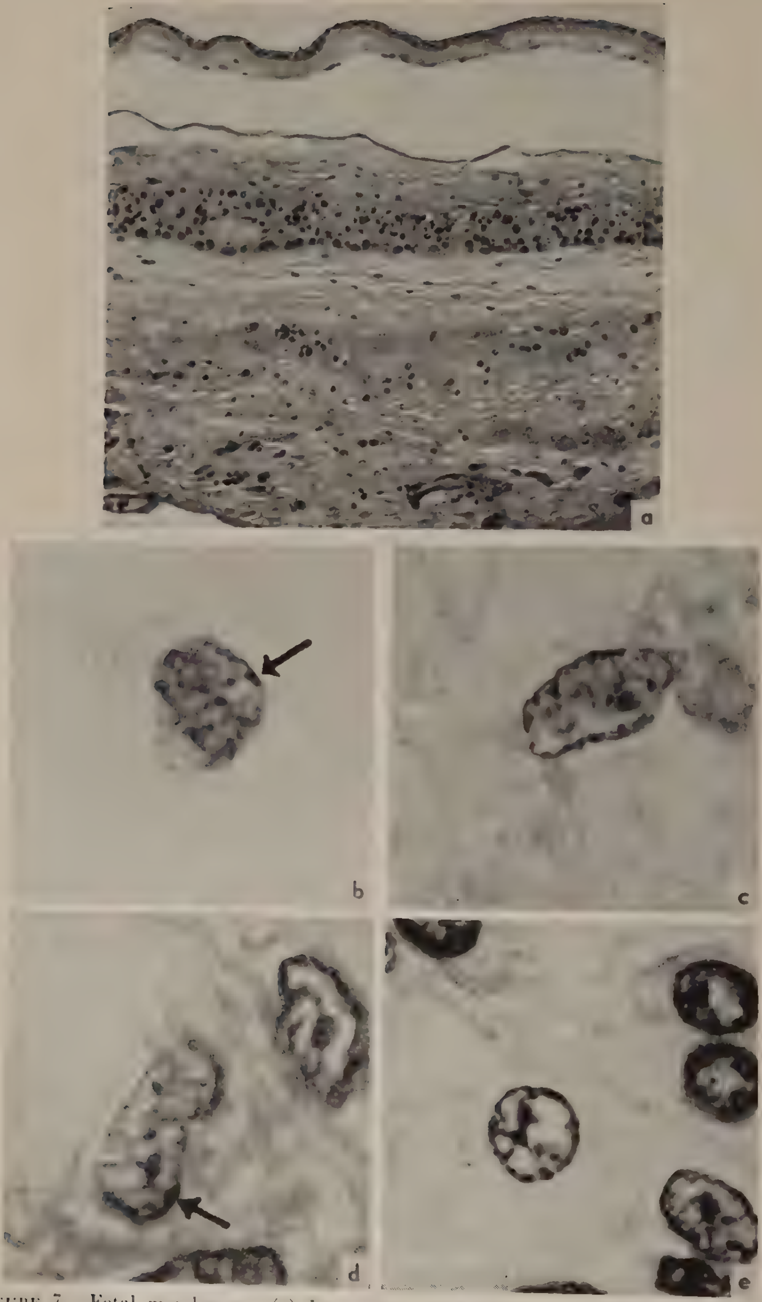

FIGURE 7. Fetal membrane. (a) Low power view showing a single layer of cuboidal amniotic epithelial cells overlying a partially separated avascular loose connective tissue layer. Adjacent is a thick layer of chorionic cells, to which some degenerating decidual tissue remains attached (H. & E., ×100). (b and c) Nuclei of connective tissue cells in layer between amnion and chorion. The cell on the left (b), containing the sex chromatin (arrow), is from the sac of a female newborn, while corresponding nuclei on the right (c), from a male infant, present no sex chromatin (H. & E., ×1600). (d) Nuclei of the chorion from a female and (e) male newborn, showing sex chromatin (arrow) only in the former (H. & E., ×1600).

ioned previously, these formalin-fixed preparations possessed fewer nuclei of dequate technical quality than did those of the larger group that had been xed in Davidson's solution. Readable nuclei were present in 6 of the male roup, and typical sex chromatin was recognized in 10 to 20 per cent of the uclei in each instance. The same incidence of nuclear sex chromatin was bserved in 13 placentas from female fetuses. Degenerative changes and indequate numbers of readable nuclei presumably accounted for the failure to emonstrate sex chromatin in the remaining female specimens.

Collections of cells with special trophoblastlike characteristics were enountered in the uterine curettings of 2 of 19 therapeutic abortions. The villar tromal nuclei indicated that one fetus was male and the other female. Typical ex-chromatin masses were observed in 10 to 20 per cent of the trophoblastlike uclei in each instance.

It thus appears that this particular type of placental cell is chromosomally emale regardless of the sex or age of the fetus.

Fetal membranes. Portions of the amniotic sac (FIGURE 7) were attached to ach of the 105 full-term placentas. The nuclei of the lining layer of cuboidal mniotic epithelial cells were almost always of poor technical quality because of ondensation, flattening, and degeneration. In contrast, the nuclei of young onnective tissue cells in the loose avascular layer between the amnion and the horion were usually satisfactory for purposes of sex chromatin identification. The nuclei of the chorion were often shrunken and vacuolated, although readble nuclei could generally be found. On the whole, the fetal membranes were ot as useful as the umbilical cord and placental tissue for nuclear sexing beause of the lack of adequate numbers of nuclei of good technical quality. Despite these limitations, complete concordance between nuclear and genital ex existed in all instances where sufficient numbers of readable nuclei were presnt (FIGURE 7).

Discussion

The foregoing observations indicate the feasibility of chromosomal sex detecion in such extrafetal products of conception as the umbilical cord, placental issue, and amniotic sac. Since these structures are derived from the developng embryo, their chromosomal sex corresponds to that of the fetus itself. Only n the placenta are there maternal components (necessarily of female chromoomal sex) which, however, can be excluded readily from the examination for uclear sex. It is thus clearly evident that with this technique manipulation of he fetus (or newborn), or even its presence, is not required for the identificaion of fetal chromosomal sex.

Certain considerations merit special comment. The estimated incidence f sex chromatin in the nuclei of chromosomal females ranged between 10 and 0 per cent. These figures are lower than those (30 to 80 per cent) recorded in he nuclei of other cells and tissues of females. Unmistakable sex-chromatin odies were not encountered in nuclei of known or presumptive male chromoomal origin. The relatively low incidence of sex chromatin in the female fetal issues studied is undoubtedly more apparent than real, and it is probably due, in large part, to technical factors. Cellular degeneration, commonly presen in placentas at or near term, significantly reduces the number of nuclei suitabl for interpretation. It may also alter the appearance of sex chromatin beyon recognition. In addition, nuclear chromatin was frequently coarsely granula and excessive in amount, so that the sex-chromatin structure may have bee obscured or even simulated. These findings necessitated the use of rigid criteri for identification of sex chromatin to the extent that only well-preserved nucle with a delicate chromatin network were employed for interpretation. A chromatin mass was not designated as sex chromatin unless it possessed th classic characteristics observed in other tissues. No allowances were mad for possible variations in size, shape, or position that might be inherent in th material under investigation.

The extension of Barr's observations to extrafetal products of conception provides a method for the prompt and facile determination of the genetic sex of fetuses and newborn patients. While other histological and cytological tests of sex (skin biopsy, oral smears) furnish the same information, the method employed in this study does not involve the fetus or newborn itself. Its routine application could be useful in the early recognition of anomalous sex development, especially in those instances in which the external genitalia may be normal. For example, it is now well known that the majority of phenotypic females with Turner's syndrome have a male sex-chromatin pattern (Polani *et al.*, 1954; Wilkins *et al.*, 1954; Sohval *et al.*, 1955). Conversely, female nuclear morphology is observed in the majority of phenotypic males with the Klinefelter syndrome (Nelson, 1955; Plunkett and Barr, 1956; Bradbury *et al.*, 1956; Sohval *et al.*, 1956). The diagnosis of these conditions could be expedited by the early demonstration of a discordance between nuclear and external genital sex. Also, the detection and classification of other types of congenital errors of sex development (such as false or true hermaphroditism) would be facilitated if and when it were found that the apparent or accepted genital sex is at variance with the chromosomal sex. This applies particularly to those patients in whom the ambiguousness of the external genitalia is so inconspicuous that the sexual anomaly may be overlooked. In this group as a whole, swift and accurate assignment of sex is of paramount importance for the patient's future sociosexual orientation.

The observations dealing with special cells situated in the basal plate and in placental septa are of considerable interest and importance. Special attention to similar accumulations of cells was paid by Wislocki (1951) and by Latta and Beber (1957). These investigators regarded them as trophoblastic elements which persist after the disappearance of the cytotrophoblastic cells of Langhans. In their opinion, these cells are distinct from typical cytotrophoblastic and syncytiotrophoblastic cells and from adjacent decidual cells. As a corollary, it was assumed that they continue with the elaboration of chorionic gonadotropin, a function that would ordinarily cease as the Langhans cells vanish.

If this interpretation is correct, these special placental cells would be derived from embryonal chorion and they would necessarily be fetal in origin. In this event, their sex-chromatin pattern should correspond to that of the fetus and other products of conception. However, the results of the present investigation indicate that typical sex chromatin was encountered in 10 to 20 per cent of the nuclei of these trophoblastlike cells regardless of fetal sex. Since they are consistently of female chromosomal origin, it is probable that they represent maternal rather than fetal components of the placenta. A recent preliminary publication by Sadovsky *et al.*, 1957, is suggestively confirmatory. In a study of the controversial matter of the composition and source of placental septa, these investigators reported a significant incidence of sex chromatin in the placental septal nuclei of each of fourteen human male fetuses at term. Accordingly, it was suggested that most of the cells of the placental septa are of maternal origin. Although the report contains no descriptions of the cells composing the septa, the cells depicted in the accompanying photomicrographs closely resemble those under discussion.

Because of their predominant location in the basal plate and their frequent extension therefrom into the substance of the placental septa, together with certain histological resemblances, it is possible that these cells represent modified decidual cells. The probable source of chorionic gonadotropin elaboration during the latter part of pregnancy remains unsolved unless it be attributed to certain chorionic cells situated in the chorionic plate on the fetal aspect of the placenta. These are usually embedded in a fibrinoid matrix and bear a resemblance to the trophoblastlike cells under discussion. Their sex-chromatin pattern invariably corresponded to the sex of the fetus or newborn.

Finally, the application of the Barr chromatin test herein described has considerable potential usefulness in problems pertaining to the sex ratio at various stages of gestation, including that of abortions. It is well known that the ratio for live-born babies is approximately 106 males to 100 females. It is also a statistical fact that the ratio for stillbirths is from 125 to 135 males for every 100 females (Ciocco, 1938) and that excess male mortality continues throughout life. Moreover, various reports indicate that, the earlier pregnancies are terminated, the higher are the ratios of males to females (Tietze, 1948, 1953). Accordingly, the conclusion has been drawn that the primary sex ratio at conception is much higher than it is among viable infants. For example, the ratio at the time of conception has been variously computed to be from 125 (Auerbach, 1912) to 160 (Martius *et al.*, 1956) and 170 (Crew, 1927) males per 100 females. This prevailing opinion has been forthrightly challenged by Tietze (1948) whose studies in more than 5700 embryos and fetuses from the third to the seventh month of pregnancy indicate a sex ratio approximating that of living newborns. There are no reliable statistics pertaining to very young embryos since gonadal differentiation does not occur until the sixth or seventh week of gestation. Moreover, until the fetus attains a crown-rump length of 50 mm., corresponding to the eleventh week of pregnancy, even trained experts cannot diagnose sex by inspection of the external genitalia (Wilson, 1926; Tietze, 1948).

Much can be accomplished toward the abolition of speculation and the resolution of controversy by the application of nuclear sex chromatin studies to the problem. In fact, sex recognition by this method in very young sexually undifferentiated human embryos has already been reported by Glenister (1956), by Park (1957), and by Witschi (1957). While embryos often are not available for examination, extrafetal products of conception usually are. Accordingly, it is reasonable to expect that further investigations along these lines will elucidate the question as to whether or not male mortality, known to be excessive during the last trimester of pregnancy and thereafter, is also excessive during the previable period of embryonic development. Such studies would be particularly useful in very early pregnancies where gonadal differentiation has not yet occurred or where the fetal sex is not discernible by inspection alone.

Summary and Conclusions

The finding by Barr and his associates that intermitotic nuclei of females contain a characteristic mass of chromatin, the sex chromatin, has already been applied to a variety of tissue components and clinical conditions. The present study consists of a systematic investigation of the nuclear sex chromatin in the various histological elements of the umbilical cord, placental tissue, and fetal membranes of the human newborn and of fetuses of different ages.

The material comprised fresh specimens obtained at the time of delivery of 55 male and 50 female apparently normal newborn infants. In addition, sections of uterine curettings of 19 therapeutic abortions performed during the sixth to the tenth week of gestation, and histological preparations of placenta and umbilical cord from 28 instances of spontaneous abortion (fetal ages 15 to 30 weeks) were studied.

Sex chromatin was identified in from 10 to 50 per cent of readable nuclei in the umbilical cords, placental tissue, and fetal membranes associated with female fetuses. It was not encountered in any of the specimens from known males. It is thus evident that the chromosomal sex of the extra-fetal products of conception corresponds to that of the fetus itself. An obvious advantage of this technique, therefore, lies in the fact that availability or manipulation of the fetus or newborn is not required for recognition of its sex.

A significant incidence of sex chromatin was encountered in the nuclei of certain placental cells regardless of the fetal sex. These special trophoblastlike cells of ill-defined origin are situated predominantly in the basal plate and placental septa where they are embedded in a fibrinoid matrix. Although they have a trophoblastlike appearance suggesting a fetal derivation, their sex-chromatin pattern indicates a maternal origin.

The routine application of this extension of the Barr chromatin test should be useful in the early recognition of anomalous sex development, especially when the external genitalia are normal or when they are so inconspicuously ambiguous that the condition may be overlooked.

The potential usefulness of the presently reported sex chromatin studies in the controversial question of the sex ratio during early pregnancy is discussed.

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APPENDIX

After this manuscript was submitted for publication, certain relevant articles came to our attention. Klinger (1957) examined 30 normal human placentas associated with 15 male and female infants. Employing whole mounts and teased preparations in some cases, as well as the Feulgen reaction using thionin as the dye, Klinger and Ludwig (1957a) reported sex-chromatin bodies in contact with the nuclear membrane in an average of 61.8 per cent of the nuclei of the amnions derived from female infants. When sex-chromatin masses lying free in the nucleoplasm or adjacent to the nucleolus were included, the incidence of sex chromatin in all extrafetal nuclei associated with female newborns ranged between 85 and 96.8 per cent. In another study Klinger and Ludwig (1957b) reported that sex chromatin was found in a significantly high percentage of nuclei of certain large cells in the placental septa associated with 2 male newborns and 3 male fetuses. It was accordingly concluded that the placental septa and the groups of large cells are of maternal rather than fetal origin. It would appear from the pictomicrographs accompanying the study that some of these cells resemble those that we have described above as trophoblastlike and that are also regarded as maternal in origin.

Considerable interest also attaches to a report suggesting that the appearance of chromocenters and, therefore, the apparent incidence of sex chromatin may be affected by altered metabolic conditions in the cells themselves (Sachs and Danon, 1956).

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Part IX. An Evaluation of Nonsteroidal Ovarian Hormones

HISTORICAL ASPECTS OF RELAXIN

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If, as biologists, we were not accustomed to the many novelties that nature continually reveals, we should be surprised at being suddenly confronted in reproductive endocrinology with the relaxin phenomenon. Relaxin is a pregnancy hormone which, though it was discovered at about the same time as the steroids, bears no structural relation to them whatever, but rather appears to be a low-molecular weight protein or polypeptide (Frieden and Hisaw, 1953). This hormone, relaxin, has had a difficult time establishing itself.

Early Developments

In 1926 F. L. Hisaw, for whom the story of relaxin represents a splendid personal achievement, embarked on a program that showed that the marked relaxation of the pelvic ligaments in guinea pigs during pregnancy is under hormonal control. The substance responsible for this effect, found in the blood of pregnant rabbits and other mammals, was able to produce the relaxation characteristic of pregnancy in virgin guinea pigs artificially brought into estrus with follicular hormone (estrogen). Extracts of corpora lutea from sow ovaries, which were also highly effective, induced various additional physiological changes, suggesting the presence of more than one hormone. Alcoholic luteal extracts soon yielded the steroid progesterone, which Hisaw helped to characterize. Aqueous fractions, on the other hand, contained the guinea pig pelvic ligament relaxing factor which, by 1930, had been isolated in some degree of purity. This substance was named relaxin (Fevold *et al.*, 1930).

During the 1930s the steroid structure of progesterone was elucidated and its therapeutic effect tested clinically, while research on its proteinlike sister hormone, relaxin, was neglected. Many investigators doubted the existence of a third ovarian hormone in addition to estrogen and progesterone, since they were able to obtain relaxation of the guinea pig pelvic ligaments (also termed pubic-symphysis relaxation) with combinations of these two steroids, or with estrogen alone. Actually, an examination of the time factor would have shown the following differences in the intervals necessary for an adequate relaxation reaction:

Treatment given	Relaxation occurs after
Estrogen alone	10 to 20 days
Progesterone, given to an estrogen-conditioned animal	2 to 4 days
Relaxin, given to an estrogen-conditioned animal	a matter of *hours*

(Adapted from Hisaw *et al.*, 1944.)

Many doubts about the individuality of relaxin were dispelled by advances in isolation and assay procedures published in 1944. The new source was defatted luteal tissue which constituted the residue in a process of progesterone extraction. This was acidified, treated with salt, neutralized, and precipitated by saturation with ammonium sulfate. One mg. of the resultant preparation, containing 15 per cent nitrogen, represented 1 guinea pig unit (GPU) of relaxin (Abramowitz *et al.*, 1944). Subsequent work on isolation of relaxin has shown that the hormone is present not only in the corpus luteum, but is distributed abundantly throughout the fresh whole ovary of pregnant sows (Albert *et al.*, 1947).

In assaying relaxin, Fevold *et al.* (1930) had designated as a GPU "the minimum amount of hormone which causes a definite loosening of the ligaments within 10–12 hours after a single injection," using virgin female guinea pigs in full estrus, and a subcutaneous route of administration. Loosening was determined by alternately moving each half of the pelvis up and down vertically while at the same time keeping a finger over the symphysis pubis. This rather inaccurate and tedious method, involving many serial dilutions, was supplanted in 1944 by that of Abramowitz and his associates, in which a series of 12 ovariectomized guinea pigs, weighing 350 to 800 gm., received estradiol for 4 days and a single relaxin injection on the fifth. The symphysis pubis of each animal was palpated manually 6 hours later. A guinea pig unit was defined as the amount of hormone that produces relaxation of the symphysis in two thirds of the 12 animals. With this method a satisfactory constant dose-response relation was obtained. In later modifications of this procedure, the degree of response of each individual animal was added to the concept of "proportion of animals responding" (Frieden and Hisaw, 1950), and a serologically interesting loss of sensitivity in repeatedly used test animals was taken into account (Noall and Frieden, 1956; Frieden, 1958).

Gradually, after 1944, animal experiments broadened the evidence that relaxin was intimately connected with pregnancy. Hisaw and Zarrow (1948) showed a rapid increase of the hormone in sow ovaries during pregnancy up to a maximum of approximately 10,000 GPU/gm. tissue when the fetus is 5 to 6 inches in length. At mid-pregnancy the value for blood was 2 GPU/ml.; for placenta, 0.5 2.5 GPU/gm. Correlation of blood levels with progress of pregnancy was shown in rabbits, in guinea pigs and, ultimately, in humans (Zarrow *et al.*, 1955).

Physiological Effects

With advances in the methods used in relaxin research during the 1940s there came new information concerning effects other than pelvic relaxation. Thus, it was first shown in 1945 that relaxin, together with small doses of estradiol and progesterone, will cause mammary gland growth and lobulation in immature ovariectomized rats. In the dosages used, all three hormones were necessary for positive results (Hamolsky and Sparrow, 1945). Mice and guinea pigs are similarly affected (Smith, 1954).

The results of intensive work during the past decade, regarding an effect of relaxin on the uterus, may be summarized as follows:

The starting material for relaxin is an aqueous extract of corpus luteum or whole ovary. Such an extract can not only cause the loosening of pubic ligaments, which is the characteristic effect of relaxin, but also can bring about a diminution of uterine contractions, that is, uterine relaxation (Krantz *et al.*, 1950; Frieden and Hisaw, 1953).

If such a crude extract is purified in a certain manner, using guinea pig pubic symphysis assays, relaxin is obtained (Frieden and Hisaw, 1950). Like the crude corpus luteum extracts, relaxin itself has been found to have a high degree of activity on both the pubic ligaments and the uterus in guinea pigs (Felton *et al.*, 1953; the work on rats of Sawyer *et al.*, 1953). However, we might ask: if relaxin, the *pubic* relaxation hormone, has a *uterine* relaxation effect, how does it differ from the purified preparations of that other nonsteroid ovarian hormone, the "uterine relaxing factor" (URF)?

On this complex problem I shall make only the following points:

(1) Relaxin is assayed for guinea pig pubic symphysis activity, while uterine relaxing factor is not. When it was thus assayed, URF showed a low degree of activity (Felton *et al.*, 1953).

(2) Chemically these two substances, which are purified by different methods, are closely related (Felton *et al.*, 1953). In 1956, Frieden and his collaborators managed, by adsorption techniques, to isolate further from purified relaxin a potent uterine-relaxing fraction that was low in relaxin activity. The major relaxin-containing fraction, however, still retained substantial URF activity. Thus we seem to be confronted with two closely related proteinlike moieties: both act on the uterus, but only relaxin has a high degree of pubic symphysis activity.

Early preparations of relaxin administered to rabbits were believed to cause an antidiuretic effect, anemia, and reduction in blood nonprotein nitrogen; these claims, however, were later withdrawn (Perkoff *et al.*, 1954).

An additional apparent effect of relaxin was found in 1952 by Frieden and Velardo, who reported inhibition of deciduomata in rats by purified relaxin preparations. In contrast with the effect in rats, deciduoma formation in mice was only slightly inhibited by relaxin, while the hormone was said to have no effect at all in the case of guinea pigs.

Still another physiological effect of this versatile hormone was the phenomenon of cervical softening, first observed in cows in 1952 and then successively in pigs, humans, rats, and mice (Steinetz *et al.*, 1957a). In their work on cervical dilatation in the sow, Zarrow and his associates (1956) point out the analogy (from the point of view of evolution, one might even say "homology") between symphyseal relaxation in guinea pigs and cervical dilatation in domestic pigs. The anatomical features involved differ considerably, but in both instances relaxin specifically facilitates the passage of the offspring at birth.

The reproductive functions of relaxin outlined here and below are remarkable for their variety. As Hall pointed out recently (1957), such variety supports "the concept that relaxin may have a more general role during pregnancy than was originally suspected." No wonder that relaxin investigators have placed increasing emphasis on the histochemical and biochemical basis of the physiological results. Research along this line has merely scratched the surface of knowledge regarding the mechanisms of relaxin action.

How does relaxin work? Particularly, what is its relation to estrogen? Microscopically, treatment of guinea pigs with estrogen alone has been observed to result in the replacement of symphyseal cartilage by loose fibrous connective tissue (Talmage, 1947; Hall, 1956). Talmage (1947) who used estrogenized animals, attributed the breakdown of both cartilage and connective tissue to the action of relaxin. Others may have thought it intellectually attractive to postulate that estrogen prepares the way by turning cartilage into connective tissue and that relaxin then takes over, simply to depolymerize the connective tissue ground substance and disaggregate the fibers (Frieden and Martin, 1954); the facts of the past several years, however, indicate a deeper, more involved mechanism. Thus, Hall (1956) has shown that relaxin alone, without estrogen, will produce some separation of the innominate bones in ovariectomized, progesterone-treated mice, even when the lack of estrogen appeared to prevent the change of symphyseal cartilage into connective tissue.

Frieden (1956) demonstrated the capacity of relaxin, again even without prior estrogen administration, to stimulate incorporation of glycine into the proteins of guinea pig symphyseal connective tissue; this protein anabolic reaction, occurring without prior estrogen, is manifestly independent of the symphyseal relaxation process. Recently Zarrow (1957) showed that relaxin can act independently of estrogen in causing an increased water uptake in the rat uterus. A further clue to the fundamental nature of the relaxin mechanism was presented by Steinetz *et al.*, who noted increases in glycogen concentration as well as in water content, dry weight, and total nitrogen in the uteri of estrogen-primed spayed rats treated with relaxin (1957b). The same group of workers found that relaxin treatment of totally ovariectomized mice maintained on progesterone, markedly increased their subsequent responsiveness to oxytocin (Steinetz *et al.*, 1957a); this observation has been reflected clinically in cases of human labor that required lessened doses of oxytocin when relaxin was given (Eisenberg, 1957).

The variety of these recent discoveries is evidence that the mechanisms of relaxation in the reproductive organs are probably more subtle and complex than a mere direct attack on connective tissue polymers by relaxin.

Clinical Effects

Clinical utilization of purified relaxin and uterine relaxing factor has followed the initial animal experiments by about a quarter of a century, if the clinical pioneering of Falls *et al.* (1942) is not counted. After the uterine relaxation experiments of Krantz *et al.* (1950), two groups of clinical workers in 1953 reported good results with aqueous extracts of sow corpus luteum in patients with dysmenorrhea and premature labor, respectively. The uterus-relaxing factor was found by these workers to appear in the blood serum within thirty minutes after oral administration (Rezek, 1953; Jones and Smith, 1954). Subsequent clinical studies by Majewski and Jennings (1955; 1957) have confirmed the efficacy of these extracts in a fair, though not spectacular, percentage of premature labor cases. Some difficulty in recognition and definition of premature labor, of course, is a recognized feature in this type of investigation.

The effect on human beings of relaxin itself was not reported until 1954, when Perkoff and his associates demonstrated the great safety of the hormone administered intramuscularly and subcutaneously. Ten subjects received amounts varying from 100 guinea pig units (GPU) to 40,000 GPU (in divided doses, over a 24-hour period). Other variables, such as leukocyte count and 17-hydroxycorticosteroids, were studied. No metabolic or physiological changes were observed in the treated persons. It is well to point out—as was done in a later publication (Zarrow *et al.*, 1955)—that these experimental subjects had not been subjected to the prior influence of estrogen, which is needed for many of the relaxin effects.

Perkoff's report, together with two articles published soon afterward in the *Journal of Clinical Endocrinology*, may be held in great part to be responsible for the current intense, though belated, interest in the medical significance of relaxin. The second paper, published by Zarrow *et al.* in January 1955, demonstrated that the concentration of relaxin in human blood serum increases progressively during pregnancy and drops precipitously at parturition; this is the sort of evidence for physiological normality of a substance that clinicians like to see. A few weeks later Abramson and Reid's paper on the use of relaxin in five patients in premature labor appeared. Publication of this pioneering study broke ground for clinical trials. Eichner (1956), Folsome (1956), McCarthy (1957), and their respective associates are among those who have contributed significant papers on the use of relaxin for uterine relaxation in threatened premature labor.

Since Eichner's use of the hormone for cervical softening in 1956, relaxin has been employed increasingly for labor at term. Using relaxin plus oxytocin, Birnberg and Abitbol (1957) reduced the length of labor by more than 50 per cent of the reduction achieved with oxytocin alone. Similarily, Eisenberg (1957) successfully used relaxin as an adjuvant in labor, both with oxytocin and alone.

The information we have on the action of relaxin would logically suggest its trial in the collagen diseases. The hormone has been tried in arthritis—where, in nonestrogen primed patients, it was found wanting (Zarrow *et al.*, 1955)—and its use in scleroderma has been reported favorably (Casten and Boucek, 1958). A spur to further efforts in this area of investigation was furnished by a report in last year's *Federation Proceedings* which indicates a direct action of relaxin on collagen tissue of rats bearing sponge implants (Elden *et al.*, 1957).

The Assay Problem

In concluding this brief historical sketch I propose to draw attention to the one problem in relaxin research that—in view of all the clinical testing now in progress—confronts us today even more emphatically than it did in years past: the problem of an adequate assaying procedure.

In standard guinea pig assays of relaxin there is, first of all, the continuing difficulty of defining the event "relaxation" that figures so importantly in any definition of a GPU. Originally, Fevold *et al.* (1930) spoke of a "definite loosening of the ligaments," as determined by manual palpation. The term "relaxation of the symphysis" used by Abramowitz *et al.* (1944) still did not convey quantitative information, although the use of twelve animals served to increase the test's statistical significance. Various changes in procedure, such as X-ray measurement, have been proposed since 1944 (Sher and Martin, 1956), yet we still experience difficulties with quantitative determination of the relaxation end point in the guinea pig assay.

A second aspect of the assay problem is the species variability of relaxin. For instance, the maximal blood titer of relaxin during pregnancy varies widely with the species; it is 10 GPU/ml. in rabbits, 2 GPU/ml. in humans and pigs, and 0.5 GPU/ml. in guinea pigs themselves (Zarrow *et al.*, 1955; Hisaw and Zarrow, 1948; Marder and Money, 1944). Striking variation existed in the apparent interaction of relaxin preparations with progesterone in experimental deciduomata; as we have seen, deciduomata were considered effectively inhibited by relaxin in the case of rats, slightly inhibited in mice, and not affected at all in guinea pigs (Frieden and Velardo, 1952). The cardinal effect of relaxin, symphyseal relaxation itself, varies with the species. In contrast to the action of relaxin on the pubic symphysis of guinea pigs, no effect of relaxin on the pubic symphysis was revealed in X-ray studies of human subjects—pregnant, as well as estrogen-primed nonpregnant individuals (Hodgkinson, 1956; Weinberg, 1956)—although a certain relaxation of the pelvic joints is a normal physiological occurrence during human pregnancy (Abramson *et al.*, 1937; Johanson and Järvinen, 1957). One species that particularly differs in its symphyseal response to relaxin is the mouse: preparations of human sera that relax the pubic symphysis of guinea pigs have been shown to be ineffective in mice (Zarrow *et al.*, 1955). On the other hand, when given in a depot form (Kliman and Greep, 1955), relaxin was shown to elicit a response in mice also.

The third and final difficulty apparent with the assaying of relaxin preparations is our ignorance of the interrelationships among the various physiological effects of this versatile hormone, quite apart from the species question. Relaxin preparations used chiefly for uterine relaxation and cervical softening at present are assayed in terms of pubic symphysis relaxing units. What assurance is there that two vials containing an equal number of pubic symphysis relaxing units will equal each other also in the number of their "cervix dilating units," or "uterine relaxation units," or "collagen anabolism units," or "mammary gland development units?"

Clearly, our new understanding of the fundamental nature of this hormone's action requires a new assay procedure which will measure its potency as a drug in terms of units more appropriate than symphyseal relaxation, in terms of units that will represent the clinical action on the uterus and cervix for which relaxin is used. Perhaps a human assay procedure can be perfected; however, an attempt to use human uterine muscle or cervical tissue for assaying relaxin failed (McGaughey *et al.*, 1958). Eventually, the increasing understanding of relaxin biochemistry will permit other advances, such as further fractionation of the hormone into chemically as well as biologically homogeneous moieties. The structure of its molecule will be elucidated and we shall be able to proceed to the precise study of the cellular reactions that form the common basis of all the physiological effects elicited by relaxin.

I trust that these remarks will be received as constructive in nature, as they are intended. Our present assay methods certainly are of use, if only as a first approximation. After all, they have brought us to the current stage of active theorizing, advanced experimentation, and clinical use. However, if the increasing relaxin studies on human subjects are to be utilized to fullest advantage, improvements in our standard assay procedures must be made.

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BIOCHEMISTRY OF NONSTEROIDAL OVARIAN HORMONES*

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The multiplicity of biological effects associated with aqueous ovarian extracts[1-7] presents a situation especially intriguing to the biochemist. In addition to the usual problems of isolation, purification, and chemical characterization involved in the study of any naturally occurring, physiologically active substance, the diversity of biological responses characteristic of such extracts also presents the question of possible chemical as well as biological heterogeneity, that is, the biochemist must determine whether more than one biologically active substance is present. An affirmative answer to this question not only complicates the problem of separation, but imposes the additional requirement that the relationships that may exist between the several compounds be defined.

This paper is concerned with the application of countercurrent distribution techniques to the problem of relaxin isolation and characterization and with the results of some experiments that bear upon the relationship of relaxin to the uterine-relaxing-factor (URF) activity of ovarian extracts. Consonant with previous usage,[8] the term relaxin is reserved for the specific agent responsible for pelvic relaxation in the estrogen-primed guinea pig; the phrase nonsteroid ovarian hormones refers to a multifunctional extract of unproved homogeneity

Distribution Studies

When a relaxin-containing extract, prepared by the salt-ethanol method,[9] was distributed between 1 per cent aqueous trichloroacetic acid (TCA) and 2-butanol, the concentration of relaxin in the organic phase was found to be about twice that in the aqueous layer, while the bulk of the protein† appeared to be equally distributed between the two phases. It was observed further that the amount of protein that appeared in the butanol layer decreased sharply when the TCA concentration was decreased. These observations indicated that countercurrent distribution methods might be very useful in separating relaxin from accompanying impurities. Therefore, an intensive study has been made of the behavior of relaxin when partitioned in 2-butanol/aqueous TCA systems.

For the experiments to be described, acetone-dried powder from the ovaries of pregnant sows was extracted by the method of Frieden and Hisaw.[9] The resulting relaxin preparations contained from 40 to 60 guinea pig units (g.p.u.)

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† As determined from the optical density at 285 mμ.

/mg. The apparatus used was a 100-tube, all glass Craig machine* equipped for automatic operation. In experiments in which more than 100 transfers were applied, the upper phase from tube 99 was recycled into tube 0. The major difficulty encountered was due to the reluctance of the 2 layers to separate rapidly and cleanly when even small amounts of protein were present; although many devices were explored in attempts to speed the separation, none was successful. It was generally necessary to operate with settling times of 30 to 60 min., and sometimes longer. This was achieved without the sacrifice of automatic operation by the use of timers† that interrupted the line current for a suitable interval during the settling phase of the cycle. Most of the experiments described here were performed in a room in which the temperature was maintained at 22 ± 1° C.

The solvents were prepared by shaking equal volumes of 2-butanol and a TCA solution of appropriate concentration. After equilibration, the 2 phases were separated, 10 ml. of lower phase were introduced into each tube, and the reservoir was filled with upper phase. The relaxin preparation was dissolved in lower phase to a concentration of 2 to 5 per cent and distributed among as many tubes as necessary, beginning with tube 1. To ensure continued saturation of both phases, tube 0 contained protein-free lower phase, and aliquots of upper phase were allowed to run ahead of the sample. At the end of the experiment the contents of each tube were removed to a graduated test tube, the volumes of the two phases were noted, and ethanol was added to a constant final volume (usually 25 ml.). Ordinarily protein concentrations were determined by measuring the optical density at 285 mμ, although in some instances, when only small amounts of material were involved, a colorimetric ninhydrin method was preferable.‡

Assays for relaxin activity were performed on groups of 7 to 12 guinea pigs by the method previously described,[9] except that, for most of the experiments to be reported, intact rather than ovariectomized animals were used. The sensitivity of each group of assay animals was tested at intervals with a standard relaxin preparation (126-1, 60 g.p.u./mg.). Assays for URF activity were performed on guinea pigs by the technique of Krantz *et al.*[10] under the supervision of H. H. Bryant of the research laboratory of Hynson, Westcott & Dunning, Inc., Baltimore, Md.

A unit of URF activity is defined as the amount that, in two thirds of the animals tested, causes a 90 per cent decrease in contraction amplitude within

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* Manufactured by H. O. Post Scientific Instr. Co., Maspeth, N. Y.

† Manufactured by Automatic Electric Mfg. Co., Mankato, Minn. A 30-min. and a 60-min. timer were used, the former permitting a settling time of approximately 28 min. and the latter of 58 min. If the 2 timers are operated in series, settling times up to 6 hours are possible. In making use of interval timers of this type, it is essential that the "on" part of the cycle coincide exactly with the time required for a complete cycle of the Craig machine, otherwise the resultant "creep" will cause irregular operation.

‡ In order to use optical methods for the determination of protein content it is necessary that the solvents used possess a low absorption in the ultraviolet. The best 2-butanol available commerically appears to be heavily contaminated with UV-opaque impurities. We have found that, by refluxing the solvent over CaO for 48 to 72 hours and redistilling, the extinction at 285 mμ can be reduced to 0.3 or lower. The corresponding "solvent blank" thereby is reduced to 0.10 to 0.15.

10 min. of its administration. An average of 9 animals was used for each sample assayed.

Figure 1 summarizes the results of an experiment in which 500 mg. of a relaxin preparation that contained 60 g.p.u./mg. was distributed for 108 transfers between 1 per cent aqueous TCA and 2-butanol. After the concentration of protein was determined in each tube, the tube contents were pooled as indicated, and the pools were assayed. Pool 4, which included tubes 61 to 85, contained about 85 per cent of the recovered activity, while pool 5, tubes 86 to 99, contained the remaining 15 per cent. Pools 1 to 3 were devoid of significant relaxin activity.

The shaded area of figure 1 is enveloped by a curve representing the expected distribution for a pure component whose distribution coefficient (K) is such that 85 per cent would be expected to remain below tube 85. The peak of this curve occurs at tube 81, corresponding to K = 3.0 (corrected for the fact that the tube that initially contained the sample was not tube 0). In several similar experiments with various relaxin preparations, values of K have ranged from 2.7 to 3.2, averaging about 3.0.

The calculation of a distribution coefficient for relaxin in these examples bears the implicit assumption that a single substance with relaxin activity is present. To test this assumption, material recovered from the active pools of 3 experiments similar to that of figure 1 were combined and then subjected

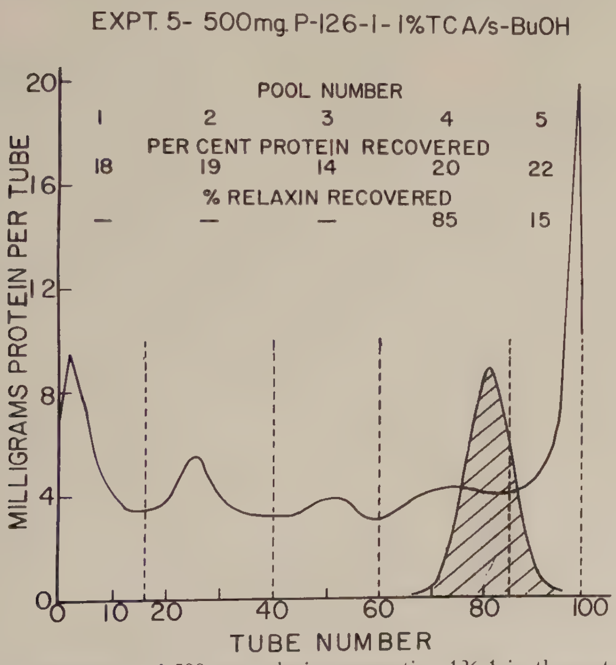

Figure 1. Distribution of 500 mg. relaxin preparation 126-1 in the system 1 per cent TCA/2-butanol.

again to 108 transfers in the same solvent system. After the protein content of each tube had been measured, single tube assays were performed on every fourth tube from tube 67 to tube 91. In experiment 9 (upper half of FIGURE 2) the open circles represent relaxin concentrations; the solid envelope of the shaded area represents the theoretical curve calculated for a substance that shows a peak concentration in tube 79 (K = 2.7). The close correspondence of the 2 indicates that but a single substance with relaxin activity is involved.

As noted above, calculated distribution coefficients for relaxin in the system 2-butanol/1 per cent TCA cluster about 3.0, and the variation from 1 experiment to another is no more than could be attributed to experimental (largely assay) error. A considerable number of experiments also has been performed with the system 2-butanol/0.1 per cent TCA. Under these conditions the distribution coefficient appears to fluctuate much more widely. Thus, in experiment 12 (lower half of FIGURE 2), which represents a 64-transfer experiment with the active material recovered from experiment 9, the interpolated activity peak corresponds to K = 0.21—the distribution of activity (open circles) again conforms to that expected for a single active substance. In other experiments, values between 0.3 and 0.4 have been more common, although their range extends to 0.75. An explanation for the volatility of the

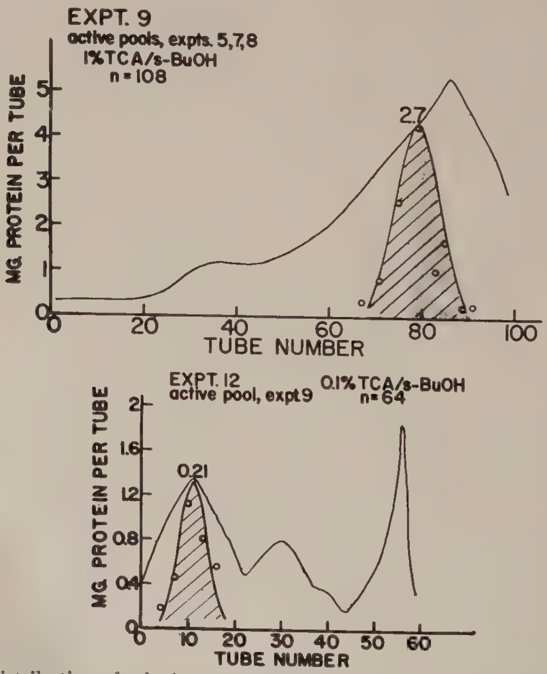

FIGURE 2. Distribution of relaxin preparations in the systems 1 per cent TCA/2-butanol (upper curve) and 0.1 per cent TCA/2-butanol (lower curve). Open circles = relaxin concentrations; solid curves = calculated distribution for components with K = 2.7 and K = 0.21, respectively.

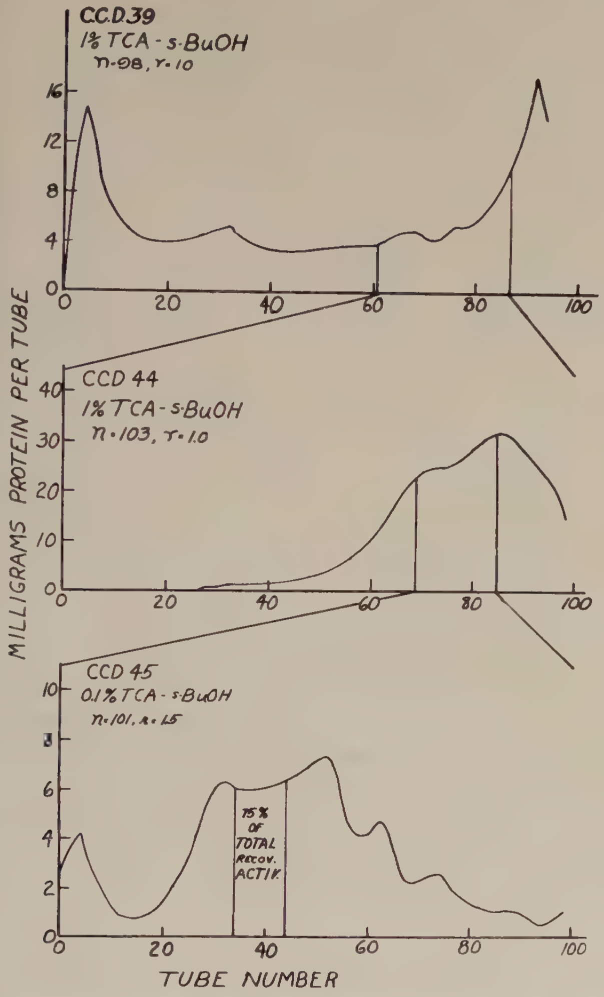

FIGURE 3. Purification of relaxin by countercurrent distribution. For details, see text.

istribution coefficient in this system has not been forthcoming. In general, ontrary to what might be expected, lower K values appear to be associated ith experiments in which the total amount of protein (and thus the protein: TCA ratio) is relatively small.

Several attempts have been made to achieve the goal of a homogeneous reaxin preparation by serial experiments using the 2 systems described above. n 1 of these (FIGURE 3),* active pools from several distributions in 2-butanol/ .0 per cent TCA (representing a total of 6.6 gm. of 40 g.p.u./mg. material) ere combined and rerun in the same solvents (experiment 44). The material

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* In FIGURES 3, 4, and 7, r is the ratio of volumes of the two phases (upper–lower).

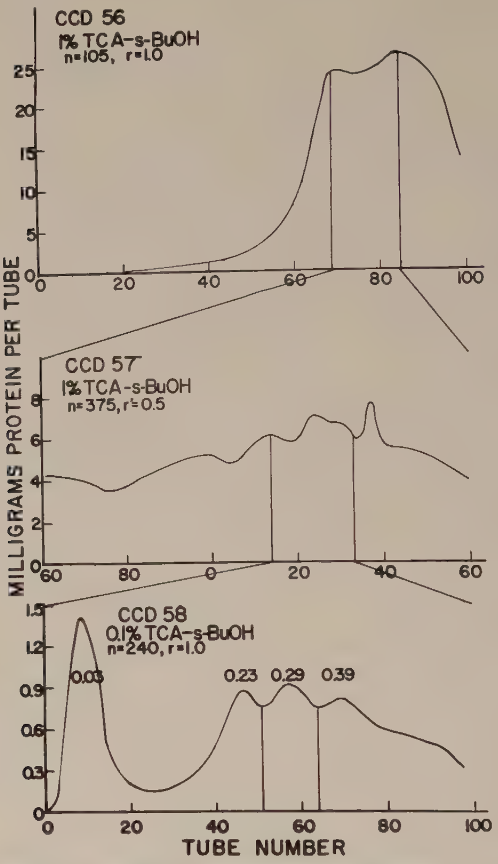

FIGURE 4. Purification of relaxin by countercurrent distribution. For details, see text.

recovered from tubes 69 to 85 was then distributed for 101 transfers in 2-butanol/0.1 per cent TCA (experiment 45). Seventy-five per cent of the activity recovered in experiment 45 was found in tubes 34 to 44 (K = 0.38). The material in these tubes, after concentrating, neutralizing, and dialyzing, possessed a relaxin potency of about 1300 g.p.u./mg., and represented an overall yield of about 25 per cent.

FIGURE 4 represents a somewhat different approach to the problem. Active pools from four 50-transfer distributions of approximately 50 g.p.u./mg. material in 2-butanol/1.0 per cent TCA were combined and rerun for 105 transfers in the same system (experiment 56). The active pool from experiment 56 was distributed between 1.0 per cent TCA and 2-butanol for a total of 375 transfers, whereupon recognizable peaks emerged (experiment 57). Although positive identification of the relaxin peak was not made, assay data indicated that the most probable K was 3.15, in good agreement with previous estimates for this ystem. When the active pool from experiment 57 was distributed for 240 ransfers in the system 2-butanol/0.1 per cent TCA (experiment 58), the egion of expected activity was resolved into 3 distinct peaks (K = 0.23, 0.29, nd 0.39, respectively). Appropriate assays indicated that the major part of he activity was to be found in the region of the middle peak. Upon paper lectrophoresis at *p*H 9.4 (when it exhibited slow migration toward the anode) s well as at *p*H 4.5 (when it exhibited rapid migration toward the cathode), his material (tubes 51 to 64) was found to consist of a single component. It ppears therefore that the substance with a distribution coefficient of 0.29 in he 2-butanol/0.1 per cent TCA system may represent the relaxative hormone n substantially pure form. The assay data, however, do not permit exclusion f the possibility that the 2 neighboring peaks (K = 0.23 and 0.39) also may ossess intrinsic relaxin activity and thus be biologically, as well as chemically, elated to the main component.

The observation that the distribution coefficient for relaxin in the system 2-butanol/1.0 per cent TCA system favors the organic layer, and decreases with decreasing TCA concentration, has been applied toward the development f an improved method for extracting relaxin from acetone-dried ovarian owder.[11] Preparations 100 to 200 times as active as the starting material, n yields averaging 90 per cent, are readily obtained.

The Relationship of Relaxin to the Uterine-Relaxing Factor of Ovarian Extracts

When relaxin preparations isolated from the ovaries of pregnant sows are ssayed for URF activity by the method of Krantz *et al.*,[11] they are found invariably to possess substantial URF activity, and a close proportionality enerally is found between relaxin potency and URF activity. In many intances, however, extracts of unselected ovaries show considerably higher URF alues than would be expected from their relaxin content. FIGURE 5, reproluced from an earlier paper,[8] includes relative potency data for 7 preparations rom unselected ovaries and 8 relaxin samples isolated from ovaries of pregnant ows. The average relaxin:URF ratio of the latter series was approximately 5 imes that of the former, and the range for all samples tested was from 1:1 to :20. Although these data are by no means conclusive, they suggest the ossibility that 2 distinct URF-active substances may be involved.

Further evidence on this point has been adduced from chromatographic tudies.[12] When relaxin preparations are chromatographed on Dowex 50 uffered to *p*H 4.5 with ammonium acetate, about 60 per cent of the protein asses through unadsorbed. This fraction contains less than 10 per cent of he relaxin activity, but may contain up to 50 per cent of the original URF ctivity. When the column is eluted with ammonium acetate at *p*H 7, the to 7 per cent of protein that emerges contains 30 to 50 per cent of the relaxin ctivity, but only 10 to 30 per cent of the URF activity.

A third fraction, which appears when the ionic strength of the *p*H 7 buffer s increased, sometimes has been found to be very high in URF, but is generally ow in relaxin. TABLE 1 summarizes the results of one such experiment. gain, the relaxin:URF ratio may be seen to vary over a twentyfold range.

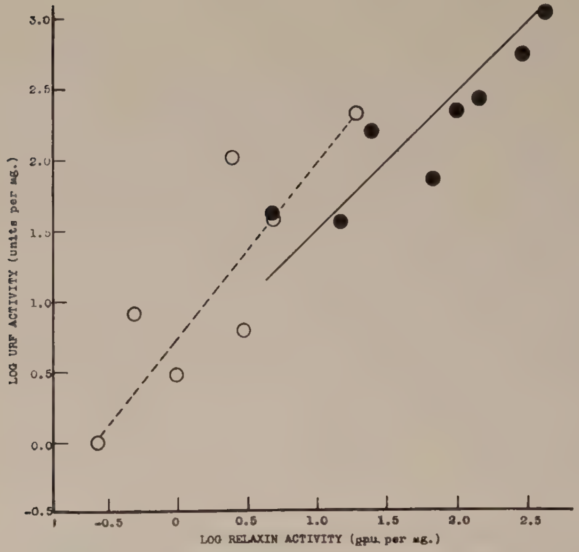

FIGURE 5. Comparison of the relaxin and URF activities of extracts of unselected (open circles) and pregnant (filled circles) sow ovaries.

TABLE 1
FRACTIONATION OF RELAXIN PREPARATION MN-4 ON DOWEX-50

Fraction number	Buffer		Per cent of total protein*	Relaxin per cent	URF per cent	Ratio R/URF
	*p*H	Ionic strength				
Original	—	—	100	100	100	1
1	4.5	0.2	60.1	<10	50	<0.2
2	7.0	0.2	5.5	35–50	10	4–5
3	7.0	1.0	1.2	<10	55	<0.2

* Determined by measuring the optical density at 280 mμ.

In the butanol-TCA systems referred to earlier, the URF activity of relaxin preparations distributes in a manner identical, within experimental error, to that of relaxin itself. Thus, in an experiment similar to that depicted in FIGURE 1, most of the URF activity was found in pool 4. Furthermore, all of th URF activity of the sample used for experiment 45 (FIGURE 3) was found in tubes 34 to 44. To explore further the relationships of relaxin and URF, comparisons were made of the partition behavior of preparations isolated from unselected ovaries, and in which the relaxin:URF ratio was relatively low. When such a preparation* was distributed for 100 transfers in the system 0.1

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* Lutrexin preparation 715B—170 URF units/mg.; 20 relaxin units/mg. Supplied by Hynson, Westcott & Dunning, Inc., Baltimore, Md.

per cent TCA/2-butanol, the observed distribution coefficients for both relaxin and URF were 0.12 (maximum concentration in tube 12); furthermore, the relaxin:URF ratio for samples representing the ascending and descending sides of the activity peak differed from each other (and from that of the original sample) by no more than 30 per cent.

FIGURE 6 shows the results of an experiment in which the same preparation (715B) was distributed between aqueous 1 per cent TCA and 2-butanol for a total of 99 transfers. The solid line represents the protein concentration, while the shaded and unshaded bars represent, respectively, the concentrations of relaxin and of URF in samples pooled as indicated. The broken line represents the theoretical distribution expected for a component with K = 3.5. The distribution of relaxin activity again conforms approximately to that expected for a single substance, although K appears to be somewhat higher than observed earlier. The distribution of URF activity, however, covers a considerably wider range than would be expected for one compound; in this experiment, relaxin:URF ratios varied over a threefold range.

FIGURE 6 suggests that there may exist in this preparation at least 2 substances with URF activity, 1 having properties very similar to those of relaxin, and another possessing a somewhat lower distribution coefficient (a third component, with K higher than 3.5, also may be present in smaller quantity). In an effort to effect a clear separation of the major components, a total of 310

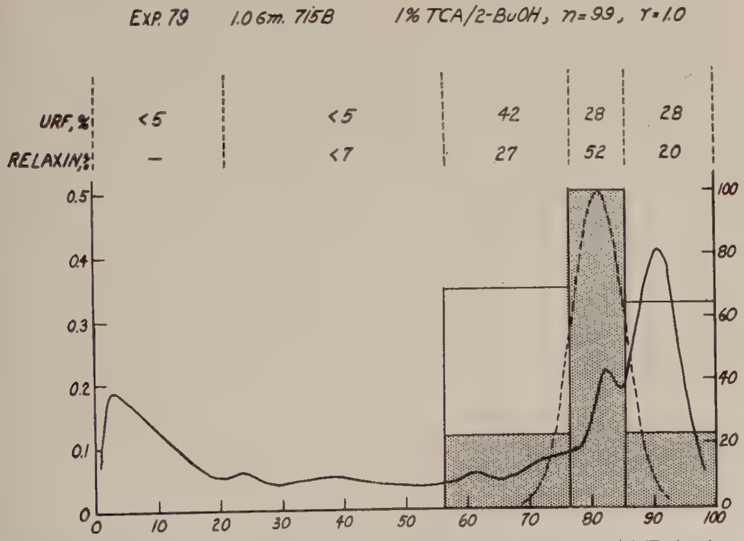

FIGURE 6. Countercurrent distribution of Lutrexin preparation #715B in 1 per cent TCA/2-butanol. The abscissa indicates the tube number; the solid line, the optical density at 285 mμ (left ordinate). Concentrations of relaxin and URF (relative to tubes 77 to 85 taken as 100) of samples pooled as indicated are shown by the shaded and unshaded bars, respectively. Percentages of recovered activity in each pool are shown at the top of the figure.

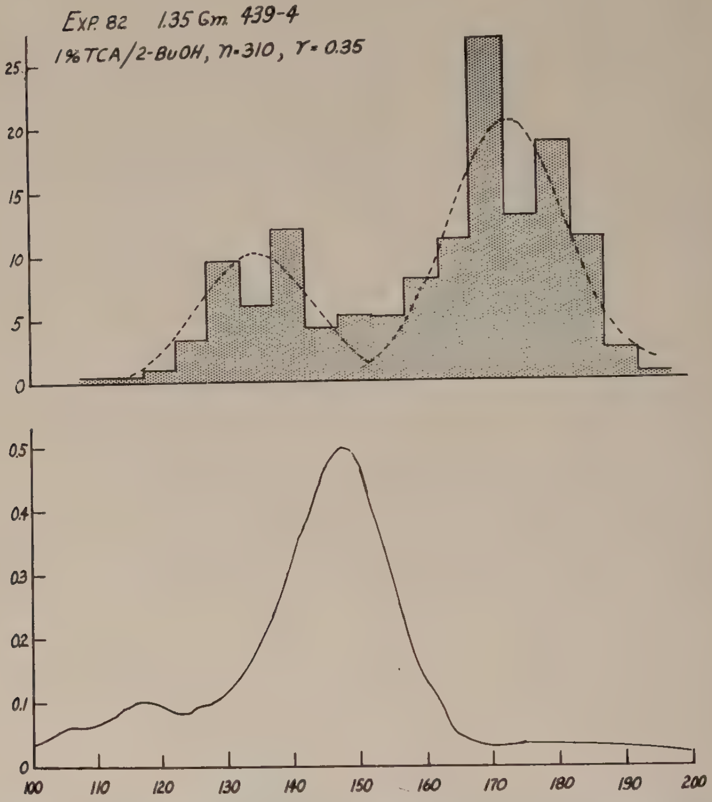

FIGURE 7. Countercurrent distribution of Lutrexin preparation 493–4 in 1 per cent TCA/2-butanol. The abscissa indicates the apparent tube number. The lower figure shows the optical density at 285 mμ; the upper figure, the URF concentrations (in units/ml.) of 5-tube pools.

transfers was applied to a preparation* similar to that used for experiment 79. For improved efficiency, the volume of the upper phase was reduced to 3.5 ml. After 100 transfers, the slow-moving component corresponding to tubes 1 to 20 in experiment 79 was withdrawn and fresh solvent was introduced into the empty tubes. The experiment was continued by recycling upper phase from tube 99 into tube 0. At the conclusion of the experiment the tubes were emptied, the volumes of each phase were recorded, and ethanol was added to 20 ml. After reading every other tube at 285 mμ, the tube contents were pooled in 5-tube lots, evaporated to 20 to 30 ml., and neutralized. Each sample was then frozen until assayed.

The results are shown in FIGURE 7. It may be observed that the URF activity of preparation 439–4 has been resolved into 2 distinct peaks. The 2

* Lutrexin preparation 439–4 -75 URF units/mg.; 3.3 relaxin units/mg. Supplied by Hynson, Westcott, & Dunning, Inc., Baltimore, Md.

broken lines indicate expected distributions for components with Ks of 2.1 and 3.6, respectively; the agreement between the expected distribution and that found by assay for URF activity must be considered good; likewise the close agreement of the distribution coefficient for the most rapidly moving URF component and that calculated for relaxin from the data of experiment 79.* The major protein component, which has an apparent maximum at tube 148, probably corresponds to the rapidly moving protein component of experiment 79; the peak tube is actually 249 (calculated K: experiment 79, 12.7; experiment 82, 10.6).

Experiment 82 indicates that the URF activity of aqueous ovarian extracts can be ascribed to the presence therein of at least two physiologically active substances. The distribution coefficient of one of these appears to be identical with that calculated for relaxin from other data. The further characterization of these compounds and the elucidation of their chemical and physiological relationships are inviting areas for future investigation.

Acknowledgment

The valuable technical assistance of Noel W. Layman and Naomi R. Stone is acknowledged.

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* Two sets of relaxin assays were performed on samples from distribution experiment 82. The first indicated that most of the relaxin activity was in the region corresponding to the second URF peak (K = 3.6). Smaller amounts were found in tubes 133 to 147 (corresponding to the first URF peak) and in tubes 148 to 162 (the interpeak region). A later assay, performed on samples thawed and refrozen, indicated that tubes 163 to 187 apparently had lost activity relative to the other 2 pools. This observation may be related to the fact that the material in tubes 163 to 187 contained very little extraneous protein, and therefore was less stable.

THE EFFECTS OF ESTROGENS, PROGESTAGENS, AND RELAXIN IN PREGNANT AND NONPREGNANT LABORATORY RODENTS

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The role of relaxin in reproductive physiological processes has been the subject of considerable investigation. However, the biological relationships of this hormone to the estrogens and progestagens are as yet poorly understood.

Treatment with progesterone and estrogen has been reported to stimulate the occurrence of a pubic symphysis-relaxing substance in the blood of ovariectomized (but not hysterectomized) guinea pigs and rabbits.[1-3] However, Hall[4] was unable to induce pubic symphyseal separation in mice with a variety of combinations of estrogen and progesterone. Indeed, progesterone has been found to inhibit relaxin-induced interpubic ligament formation in mice.[5-8]

Relaxin has been reported to have a synergistic action with estrogen and progesterone in stimulating development of mammary glands of mice and rats,[9-11] and in maintaining pregnancy in spayed mice.[12]

Despite the convincing findings that relaxin acutely inhibits spontaneous uterine motility *in vivo* and *in vitro* in a variety of species,[13-18] there is increasing evidence that the hormone may play an important role in spontaneous or oxytocin-induced delivery in mice.[8, 12, 19, 20] Crelin's[21] discovery that mechanical prevention of the normal separation of the pubes (and thus prevention of enlargement of the diameter of the birth canal) did not interfere with parturition in intact pregnant mice suggested that there are important target organs for relaxin other than the symphysis pubis.

Several investigators have reported that relaxin induces softening of the uterine cervix,[22-27] and this effect may be associated with changes in cervical water content and mucopolysaccharides.[25]

Rapid increases in uterine water content[28-30] nitrogen, and glycogen[29] have been reported following relaxin administration to immature or estrogen-primed spayed rats.

Because the uterus and cervix of pregnancy are normally subjected to the entire ovarian complex, it seemed important to explore the possible relationships of relaxin to the actions of estrogen and progesterone in pregnant as well as nonpregnant laboratory animals. In addition to crystalline progesterone, the synthetic steroid 17α-ethyl-19-nortestosterone was used in some of the experiments, as preliminary observations indicated that it was five to ten times as active as progesterone in maintaining pregnancy in spayed rats.

MATERIALS AND METHODS

The relaxin-containing extracts used in these experiments were bioassayed by two and, in some cases, three different methods: (1) palpation of guinea pig pubic symphysis; (2) direct measurement of mouse interpubic ligament; and (3) inhibition of spontaneous motility of estrous mouse uterine horns *in vitro*. The methods were in general good agreement and the activity was expressed in

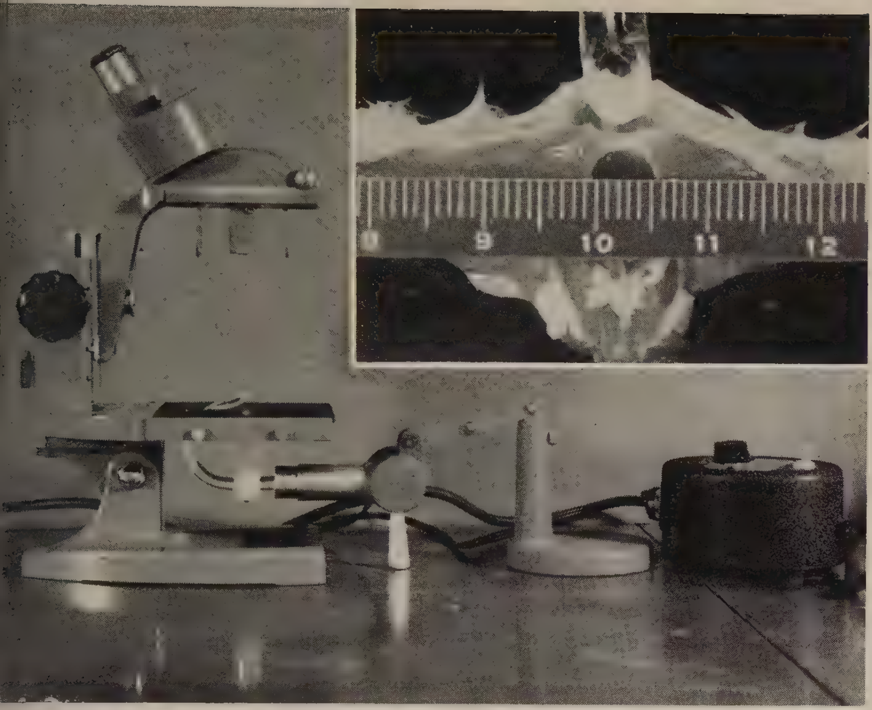

FIGURE 1. Dissecting scope equipped with fixed transilluminating device and ocular micrometer for measurement of interpubic ligament length in mice. Inset shows the ventral view of a dissection of a mouse pelvis, showing the interpubic ligament positioned on the transilluminator.

TABLE 1

BIOASSAY OF RELEASIN CONTROL PREPARATION W1164-3, LOT 016, VIALED SOLUTION (U) PREPARED FROM A SAMPLE OF REFERENCE STANDARD POWDER, 20 MG./ML. MOUSE INTERPUBIC LIGAMENT METHOD: REFERENCE STANDARD (S), (LAB. # W1164-A, LOT 8) TESTED IN PARALLEL ON THE SAME DAY

All Doses Administered S.C. in Volume of 0.2 ml. 1 Per Cent Benzopurpurine 4-B

Preparation	No. mice	Dose /mouse	Average lig. length, mm.	S.E.
S	21	1.67 μg.	1.29	0.11
S	20	3.33 μg.	1.65	0.18
S	19	6.67 μg.	2.51	0.14
U	19	$^1/_{12000}$ ml.	1.19	0.10
U	21	$^1/_{6000}$ ml.	1.78	0.18
U	20	$^1/_{3000}$ ml.	2.73	0.14

Statistical analysis

Slope b = 2.29	Potency of lot 016		Limits of error Percentage
Index of precision	mg./S/ml.	range p = 0.95	p = 0.95
λ(s/b) = 0.28	21.6	17.0–27.4	79–127

TABLE 2

BIOASSAY OF RELAXIN PREPARATIONS USING A REFERENCE STANDARD

Comparison of Guinea Pig Pubic Symphysis Palpation and Mouse Interpubic Ligament Measurement Methods

Material and method of assay	Total number of animals standard (S) + unknown (U)	Potency of unknown Mg. S/Ml. or Mg.	Limits of error Percentage p = 0.95	λ
M 30-31 Powder				
Guinea pig	85	0.74	77–131	0.27
Mouse	107	0.74	78–128	0.26
M 66 Powder				
Guinea pig	120	1.06	76–131	0.33
Mouse	140	0.99	72–140	0.33
Lot 016 vial (prep. from std.)				
Guinea pig	120	17.4	73–137	0.40
Mouse	120	21.6	79–127	0.28
Lot 017 vial				
Guinea pig	80	19.0	73–138	0.41
Mouse	97	22.2	78–128	0.26
M 36 Powder (low potent)				
Guinea pig	110	0.07	66–151	0.47
Mouse	69	0.07	81–123	0.21
Lot 018 vial (low potent)				
Guinea pig	60	1.72	70–144	0.40
Mouse	104	1.70	76–132	0.30

terms of milligrams equivalence to our own laboratory relaxin reference standard designated as W1164-A, Lot 8.[a] Detailed descriptions of the methods and analyses of bioassay data are to be published elsewhere. However, brief descriptions and illustrative data are presented as background information for the multiple hormone experiments.

Mouse interpubic ligament method. Immature albino female mice, 18 to 21 gm., were primed with a single subcutaneous injection of 5 μg. estradiol cyclopentylpropionate[b] on day 0. On day 7, "unknowns" and the relaxin standard were dissolved in 1 per cent benzopurpurine 4B, also referred to as L-390, an azo dye repository agent[c],[29] and injected subcutaneously. The relative ineffectiveness of relaxin in water or saline when tested for symphyseal effects in

[a] Warner-Chilcott relaxin reference standard, assaying 150 g.p.u./mg. by old criteria. The standard has been referred to in the literature by its old laboratory number B37-348/8a1 alpha (see Miller *et al.*[16] and McGaughey *et al.*[58]

[b] Estradiol cyclopentylpropionate (Depo Estradiol). The Upjohn Company, Kalamazoo, Mich.

Progesterone, U.S.P. and estradiol-17β, U.S.P. Syntex, Chemical Specialties Company, Inc., New York, N.Y.

17α-Ethyl-19-nortestosterone (Nilevar, production lot MA29, stated to be 100 per cent pure). G. D. Searle & Co., Chicago, Ill.

Repositol Progesterone. Pitman Moore Company, Division of Allied Laboratories, Inc., Indianapolis, Ind.

[c] Supplied by R. G. Sanders, Lambert-Hudnut Division of Warner-Lambert Pharmaceutical Company, Morris Plains, N. J.

TABLE 3

BIOASSAY OF RELEASIN CONTROL PREPARATION W1164 3, LOT 016, VIALED SOLUTION (U) PREPARED FROM A SAMPLE OF RELAXIN REFERENCE STANDARD POWDER, 20 MG./ML. GUINEA PIG PUBIC SYMPHYSIS PALPATION METHOD. REFERENCE STANDARD (S) (LAB. # W1164-A, LOT 8) TESTED CONCOMITANTLY ON FOUR ASSAY DAYS WITHIN A NINE-DAY PERIOD*

Preparation	No. pigs	Dose/pig	Average score	S.E.	Median score	Percentage Pos.
S	9	1.67 μg.	0.78	0.38	0.5	5
S	40	3.33 μg.	1.53	0.26	1.0	20
S	40	6.67 μg.	3.49	0.24	4.3	71
S	20	13.33 μg.	4.13	0.30	4.5	85
U	10	1/12000 ml.	0.30	0.12	0.0	0
U	30	1/6000 ml.	1.68	0.30	1.4	20
U	30	1/3000 ml.	2.59	0.39	4.0	57
U	20	1/1500 ml.	3.88	0.36	4.5	83

Statistical analysis (based on scores only):

Slope b = 4.07	Potency of Lot 016		Limits of error Percentage
Index of precision	mg. S/ml.	Range p = 0.95	p = 0.95
λ(s/b) = 0.395	16.2	13.4–20.8	77–129

* See Table 2 for statistical evaluation of a second assay on this preparation.

mice has been reported.[31] Necropsy was performed 18 to 24 hours later, the symphyseal area exposed, and the interpubic ligament measured directly at its shortest points with a calibrated micrometer eyepiece at a usual magnification of × 13. Visualization with the binocular microscope was facilitated by trans-llumination of the ligament (figures 1*a* and 1*b*) with a lucite rod and light source after removal of rectum, vagina, and surrounding connective tissue. TABLE 1 lists the responses obtained with a control preparation made from a sample of the reference standard processed into a sterile-filtered and vialed aqueous saline solution containing a preservative. Comparison with the bioassay statistics of five other lots is shown in TABLE 2. This mouse interpubic ligament method for the assay of relaxin was proposed by the authors[32] as an objective, practical, rapid, and reliable method for the bioassay of relaxin preparations when a minimum of 60 to 120 mice are employed and equally divided between reference standard and "unknown" at 3 or more dosage levels. With this assay the limits of error at the 95 per cent confidence level are usually within ±20 to 40 per cent.

Guinea pig pubic symphysis palpation method. This method, which has been previously proposed,[33] represents a considerable departure from earlier pro-edures.[34, 35] Intact virgin pigs, 350 to 400 gm., were estrogen-primed with 5 μg. estradiol cyclopentylpropionate S.C. on day 1 and relaxin-primed (20 μg. Standard, W1164-A, Lot 8, in saline) on day 5 each week until a "positive" esponse, at palpation 6 hours after injection, was observed. Usually 2 to 4 weeks were required to achieve this response in the majority of animals. Only pigs that were positive the previous week were considered eligible for assay. "Negative" animals from each assay were segregated for priming with estrogen and relaxin the following week. Assay animals responding "positively" were reprimed only with estrogen. Samples and dilution-dosages were coded, and palpation of the pelvis to assess mobility at the symphysis was done by two persons whose observations were separately recorded. The subjective evaluations of each palpator were scored on an arbitrary scale of 0 to 6, a score of 4 or more being considered "positive." Statistical analyses were based on the scores and calculated according to the methods of Bliss[36] and Snedecore.[37] The median score was frequently calculated, but served only as a check on the average score, which was used to make tentative potency estimates. TABLE 3 illustrates the data for the processed, vialed, solution of the reference standard versus the standard, the same preparation which was assayed by the mouse interpubic ligament and detailed in TABLE 1. Comparison with the statistical evaluation of the data on 6 lots of "regular" and control solutions and powders is shown in TABLE 2.

Mouse uterine motility inhibition method. This test was devised after confirming the original report[13] that relaxin inhibited spontaneous uterine motility *in vitro*. Uterine horns from virgin young adult mice approximately 10 weeks old and exhibiting cornified vaginal smears and uteri that had passed the ballooned stage were attached to an isotonic lever in a constant temperature bath of 25 or 50 ml. of modified Ringer's at approximately 37° C. Aeration was with either O_2 or 5 per cent CO_2 to 95 per cent O_2. The bath containing one horn received the reference standard and the mate horn received the "unknown." After an initial control period of 10 to 15 min., dosages were added to each bath at 5 min. intervals and in increments that doubled the concentration in the bath, for example, 0.1, 0.1, 0.2, 0.4 ml. Optimal concentration of the standard stock test solution was 6.7 μg./ml. of saline. Best results were obtained when the first effect, that is, reduction in height of contractions, began after the second or third addition to the bath (FIGURE 2). With unknowns it was necessary to make trial dilutions and tests to determine the optimal concentration for obtaining an effect, if any, from the second to the fourth addition to the bath. To determine relative activity the minimal total dose for similar degrees of motility amplitude inhibition, for example, 50 per cent was determined and the quotient S/U calculated. Good test recordings from 4 to 8 pairs of uterine horns with one or more dilutions were usually necessary for consistent results. Analysis of data obtained by this method will be published separately. There was generally good agreement (±50 per cent) between this and the pubic symphysis methods.

An *in vitro* method of relaxin bioassay employing rat uterine segments has been proposed by Wiqvist and Paul.[18] By their method a low potent control extract was found to be one fifteenth the potency of the reference standard used in these studies, exactly confirming mouse and guinea pig pubic symphysis assays done in our laboratories on the same preparations.

Comparison of assay methods. Both of the pubic symphysis methods require

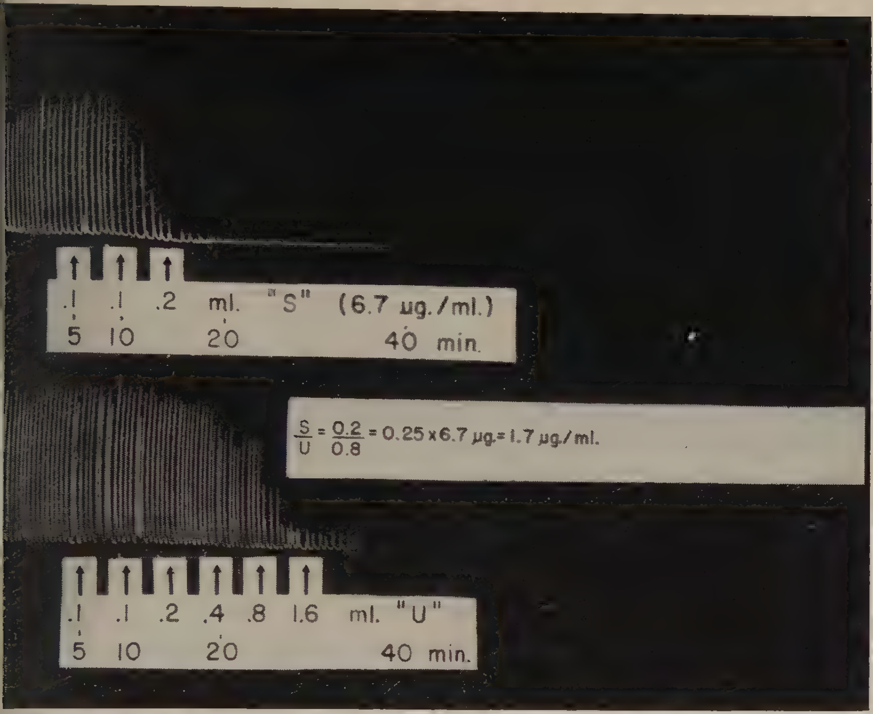

FIGURE. 2. Kymographic tracings of paired horns of mouse uterus treated with increasing dosages of Standard (S) and Unknown (U) preparations in 25 ml. bath.

similar numbers of animals and dosage levels for limits of error under ±50 per cent at the 95 per cent confidence level. Generally the limits of error are lower and the index of precision better for the mouse symphysis method. It has an objective end point, quickly and accurately measured. While the guinea pig palpation method is subjective, tedious, and requires time to learn, it has limited but definite value as a research tool on a small scale basis. When a highly effective repository agent such as L-390 is employed in the mouse ligament assay, the dosages for "mid-point of the curves" responses are similar in both tests, that is, 2 to 4 µg. However, a larger total dose can be administered to the pigs so that the sensitivity of the guinea pig assay may be three- or fivefold greater depending upon the material, for example, serum or extract fractions being tested. The estrous mouse uterine segment method provides a different biological end point and is useful as a rapid screening test.

Since relaxin has not yet been isolated as a pure hormone, adequate and objective bioassay information on all relaxin-containing materials to be used experimentally or clinically is considered absolutely essential to the interpretation of results.

Animals and Operations

Nonpregnant female rats (Wistar)[d] were ovariectomized when 31 to 38 days of age and allowed a 2-week recovery period before use.

Pregnant rats (Wistar),[e] obtained on the thirteenth day of gestation, were ovariectomized on the fifteenth day.

Immature (19 to 21 gm.) female albino mice[f] were ovariectomized for some experiments and allowed at least a 2-week recovery period.

Pregnant mice (CFW)[g] were obtained when 15 days pregnant. Unilateral or bilateral ovariectomy or sham operation was performed on day 16 of gestation.

Preparation of Hormones

Steroid hormones[b] were dissolved in sesame or Mazola oil and injected in 0.1 to 0.2 ml. volumes. Dosages are indicated in the text.

Relaxin preparations were administered as a suspension in 0.1 ml. 5 per cent beeswax in peanut oil,[32] as a solution in 1 per cent benzopurpurine 4-B (L-390[c]), or in saline solution. The following Releasin purified powders were employed: W1164-A, Lot 8[a] (Relaxin Reference Standard), W1164-A, Lot 5-7 (1 mg.S/mg.), B37-151/6a (0.3 mg.S/mg.) and B37-218/1LA1α (1.33 mg.S/mg.). All dosages were administered on the basis of bioassay activity in terms of standard potency as indicated in the text.

Placebos consisted of the appropriate vehicles in each experiment.

Oxytocin[h] was injected at a dosage of 0.5 I.U. in 0.25 ml. 20 per cent PVP solution.

Determinations

Glycogen and water contents of tissues were determined as previously described.[29] Because of the differences in glycogen concentration at or between implantation sites of pregnant uteri, only segments (approximately 200 mg.) directly over placentae were used for determination. Such tissue allowed comparison with placental glycogen and made possible the detection of a gradient across the placenta. Glycogen values were expressed as micromoles glucose equivalents per gram fresh tissue, and water content was expressed as a percentage of uterine wet weight.

Cervical Dilatability

This factor was measured as previously described.[8]

Calculations

Results are expressed in terms of mean values and their standard errors.

Because of the large variations in litter size occurring in pregnant animals, results dealing with fetal survival, births, and abortions are generally expressed in terms of the average percentage of each litter[8] rather than average number of fetuses per mother.

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[d] Purchased from the Budd Mountain Rodent Farms, Chester, N. J.

[e] Purchased from the Charles River Breeding Laboratories, Wilmington, Mass.

[f] Purchased from A. J. Barckman and Son, Barnegat, N. J.

[g] Purchased from the Carworth Farms, New City, N. Y.

[h] Pitocin. Parke, Davis & Company, Detroit, Mich.

A glycogen deposition index was calculated as an adjunct to glycogen *concentration* values, as it was found that deposition could occur without reflecting a

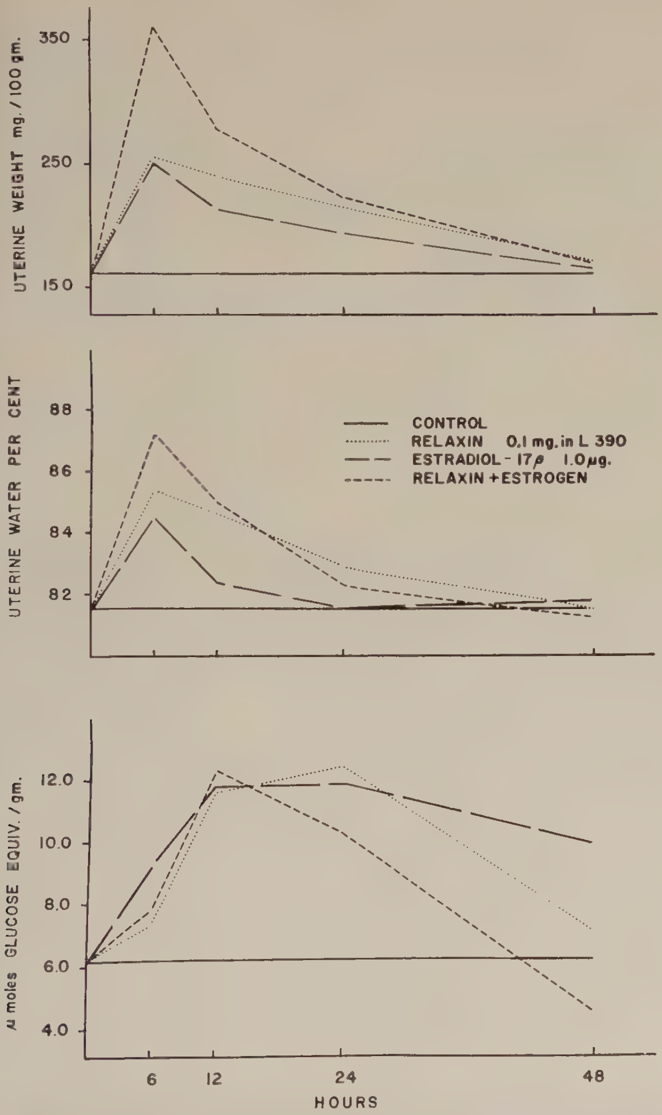

Figure 3. Effects of single injections of relaxin, estradiol-17β, or their combination on uteri of ovariectomized rats primed 1 week previously with 5 μg. estradiol cyclopentylpropionate. The dosages of relaxin and estradiol were given at zero hour. Controls injected only with the vehicle were sacrificed at identical time intervals. Average values of 4 to 9 rats were used for each point.

change in glycogen concentration, that is, when uterine weight increase parallels glycogen deposition.

$$\text{Glycogen deposition index} = \frac{\text{glycogen per uterus experimental}}{\text{glycogen per uterus control}}$$

An index greater than 1 indicates glycopexis; an index less than 1 indicates glycogenolysis.

Results

Effects of a Single Injection of Relaxin on Uteri of Estrogen-Primed Rats and Mice

A single injection of 0.1 mg. relaxin in 1 per cent L-390 (benzopurpurine 4-B) induced a rapid but relatively transient rise in uterine weight, water, and

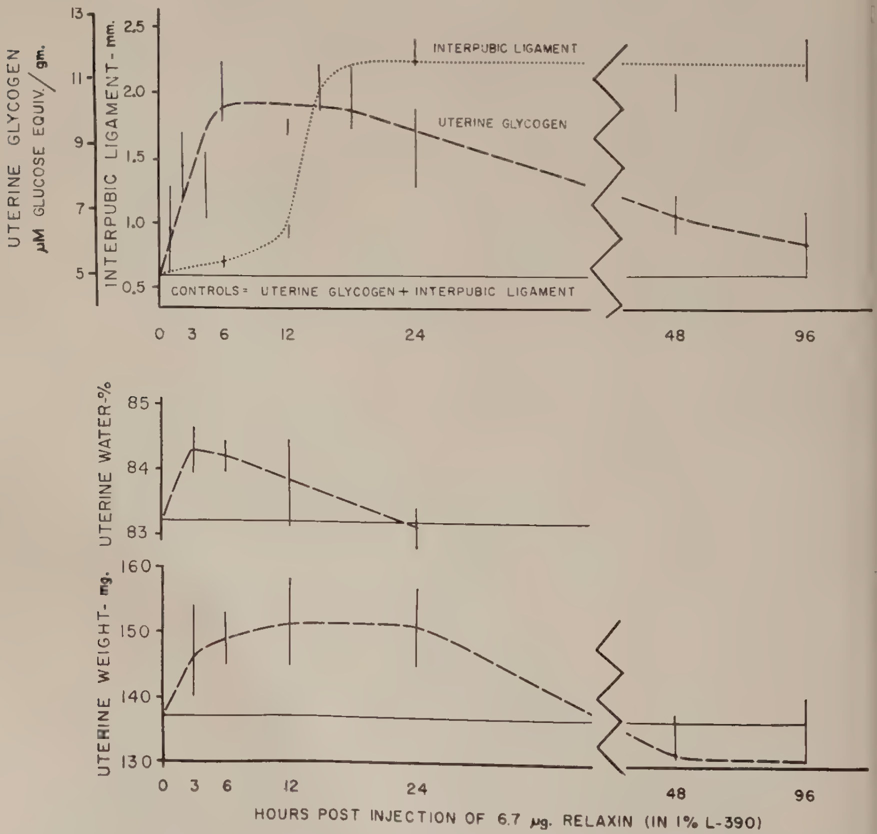

FIGURE 4. Effects of a single injection of relaxin in L-390 on uteri and pubic symphyses of immature mice primed 1 week previously with 5 μg. estradiol cyclopentylpropionate. Five to 20 mice were sacrificed at each time interval postinjection. The thin horizontal lines represent averages of concomitantly run placebo-injected controls. The thin vertical bars indicate standard errors of means.

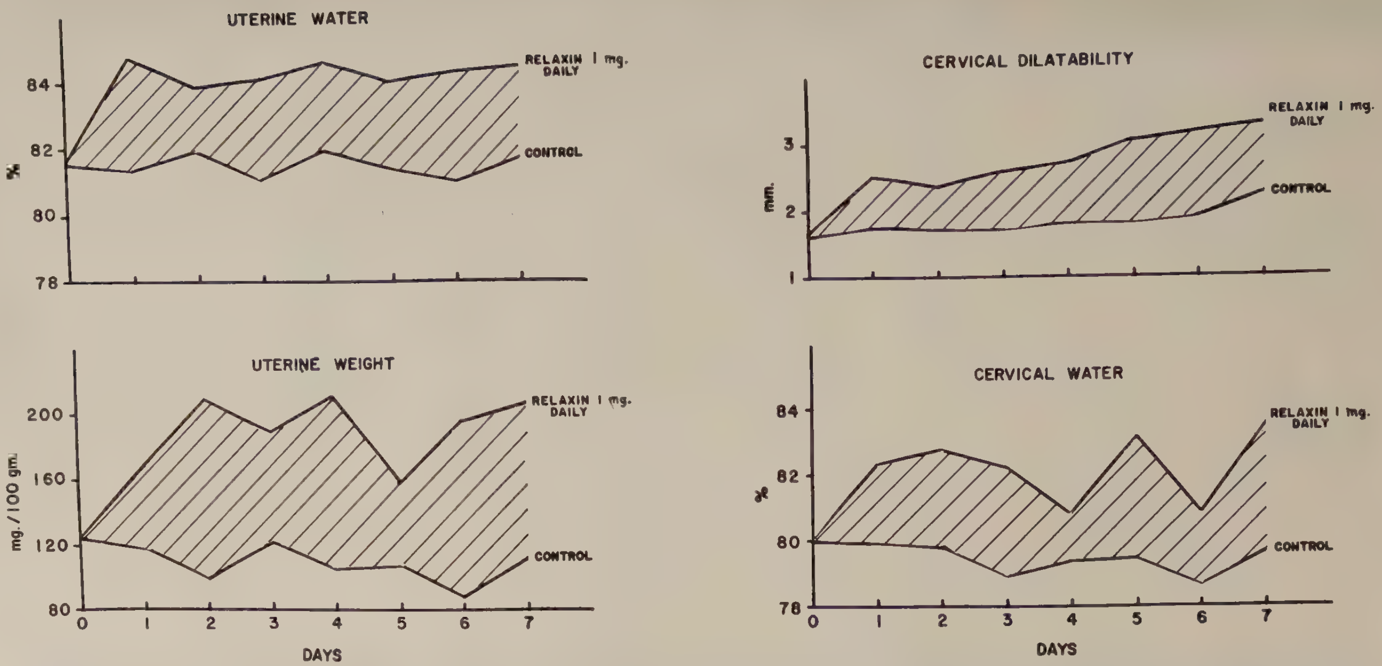

FIGURE 5. Effects of multiple injections of relaxin in a beeswax-oil vehicle on uteri and cervices of rats primed 1 week previously with 5 μg. estradiol cyclopentylpropionate. Four to 12 experimental and control rats were sacrificed at daily intervals following 1 to 7 injections of relaxin or placebo. The shaded area represents the difference of the 2 response curves.

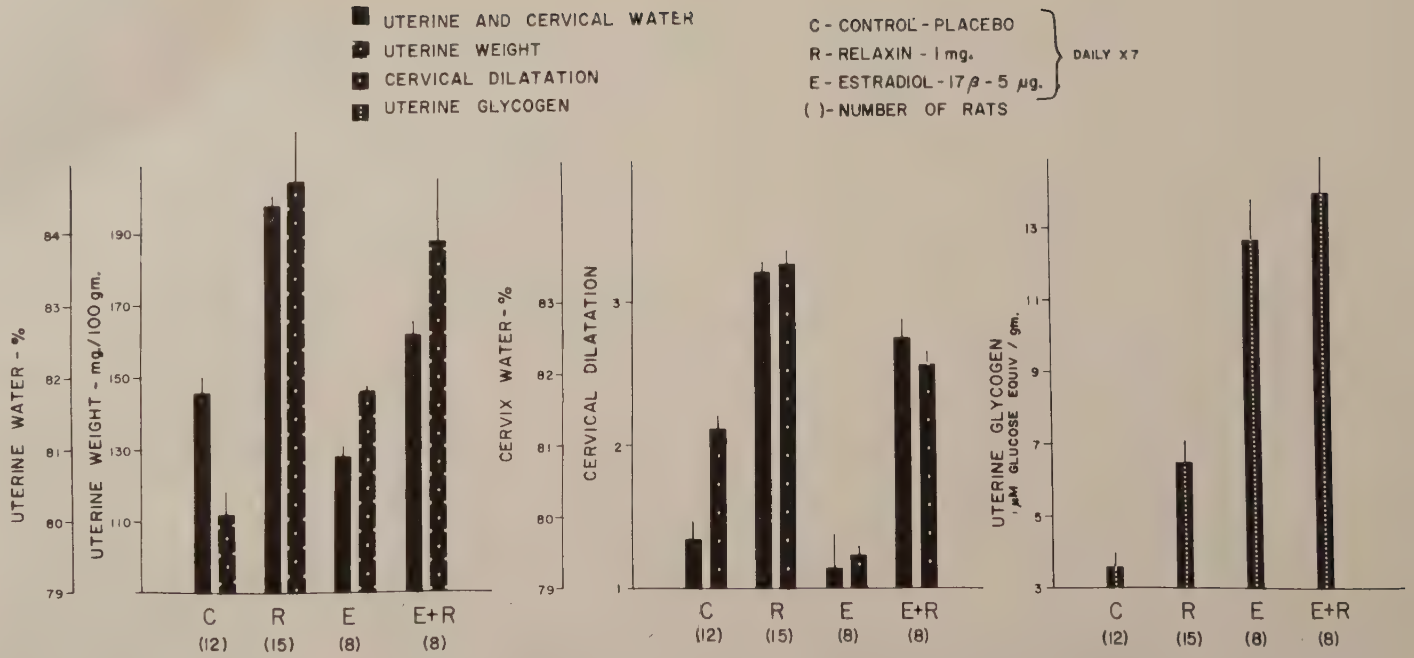

FIGURE 6. Effects of 7 daily injections of relaxin in beeswax oil and/or estradiol 17β in oil on uteri and cervices of ovariectomized rats primed 1 week previously with 5 μg. estradiol cyclopentylpropionate. Similarly primed control animals received 7 daily injections of the vehicles. The thin vertical lines show 1 standard error.

glycogen concentration in estrogen-primed spayed rats (FIGURE 3). Similar results were obtained with a single injection of 1 μg. estradiol-17β, and when both hormones were given concomitantly, augmentation of the uterotrophic and hydrating effects was observed (FIGURE 3).

In immature (intact) estrogen-primed mice, a single injection of 6.7 μg. relaxin induced a rapid increase (6 hours) in uterine glycogen, but uterine weight and water content were only slightly elevated (FIGURE 4). In contrast, no significant effects on the interpubic ligament were observed to occur before the ninth to twelfth hour postinjection. Most rapid separation of the pubes occurred between the twelfth and fifteenth hour after treatment. Uterine effects were not demonstrable after 48 hours had elapsed, but the interpubic ligaments showed no signs of regression at the ninety-sixth hour (FIGURE 4).

Effects of Multiple Injections of Relaxin, Estrogens, and Progestagens in Spayed Estrogen-Primed Rats

When spayed rats, primed day 0 with 5 μg. estradiol cyclopentylpropionate (ECP), were treated with daily injections of 1 mg. relaxin in a beeswax oil vehicle from days 8 to 14 and sacrificed at daily intervals, comparison with concomitant controls revealed: (1) a rapid rise in uterine weight and water content, (2) maintenance of increased uterine weight and water, (3) a more gradual increase in dilatability of the uterine cervix and an elevated cervical water content (FIGURE 5).

Similarly primed spayed rats treated with 5 μg. estradiol-17β daily from days 8 to 14 and sacrificed on day 15 revealed some increase in uterine weight, a decreased uterine water content, decreased cervical dilatability, and an increased uterine glycogen concentration. Furthermore, administration of the estrogen and relaxin (1 mg. daily in beeswax oil) concomitantly resulted in a diminution of the effects of relaxin on uterine and cervical water and cervical dilatability (FIGURE 6). No augmentive effect on uterine glycogen was observed.

Similar effects of increasing estrogen on relaxin response were observed when the priming dose was raised from 5 to 50 μg. ECP. Accordingly, the following experiments were simplified by comparing the effects of multiple injections of relaxin and progestagens in spayed rats (1) unprimed, (2) primed with 5 μg. ECP, (3) primed with 50 μg. ECP.

Factors Influencing Uterine Composition

Effects of estrogen priming. Uteri of rats primed with 5 μg. ECP on day 0 that received daily placebo injections from days 8 to 14 were fivefold heavier than uteri of placebo-treated, nonestrogenized animals. Uterine water content was slightly higher in the estrogen-treated group, but glycogen concentration was not different in uteri or cervices of the two groups. When the priming dose was increased to 50 μg. ECP, uterine weight (wet and dry) was further increased. There was no further increase in water content, but uterine and cervical glycogen concentrations were markedly elevated (TABLE 4, FIGURE 7).

TABLE 4
EFFECTS OF RELAXIN, PROGESTAGENS AND ESTROGEN ON THE UTERUS OF THE SPAYED RAT

Estrogen prime	Treatment[c]		No. rats	Uterine weight mg./100 gm.		Percentage water uterus	Uterine glycogen μ moles glucose equiv. per gm. fresh	Uterine glycogen deposition index	Cervix wet weight mg./100 gm.	Cervix glycogen μ moles glucose equiv. per gm. fresh	Cervical dilatability mm.
	Relaxin	Progestagen		Wet	Dry						
Five μg. ECP controls[b]			8	125 ± 5[d]	23.0 ± 1.5	81.6 ± 0.28	7.8 ± 0.9	—	27 ± 1	8.8 ± 0.9	1.66 ± 0.10
	Placebo		12	112 ± 3	19.7 ± 1.1	81.8 ± 0.21	3.6 ± 0.4	1.00	26 ± 2	5.4 ± 1.2	2.11 ± 0.07
	0	P[e] 1 mg.	12	114 ± 7	19.6 ± 0.9	82.5 ± 0.06	3.2 ± 0.4	0.89	20 ± 2	4.2 ± 0.6	1.50 ± 0.03
	0	P 5 mg.	8	88 ± 4	16.6 ± 1.1	80.6 ± 0.26	6.7 ± 1.0	1.46	17 ± 1	7.7 ± 0.9	1.62 ± 0.05
	0	ENT[f] 0.1 mg.	8	149 ± 9	23.1 ± 0.4	84.3 ± 0.31	3.5 ± 0.5	1.29	29 ± 2	5.7 ± 0.6	1.91 ± 0.08
	0	ENT 1.0 mg.	5	183 ± 12	29.0 ± 1.4	84.0 ± 0.38	6.7 ± 0.5	3.20	37 ± 1	6.1 ± 1.2	2.20 ± 0.05
Five μg. ECP[a]	1 mg.	0	9	205 ± 7	31.6 ± 0.9	84.4 ± 0.14	6.5 ± 0.6	3.31	53 ± 4	8.6 ± 0.6	3.26 ± 0.08
	1 mg. (saline)*	0	9	142 ± 11	23.6 ± 1.2	82.9 ± 0.14	8.5 ± 0.7	3.03	32 ± 2	8.5 ± 1.0	2.05 ± 0.08
	1 mg.	P 1 mg.	14	216 ± 6	33.6 ± 1.6	84.3 ± 0.14	5.1 ± 0.6	2.74	56 ± 4	7.7 ± 0.6	3.50 ± 0.10
	1 mg.	P 5 mg.	10	185 ± 17	30.5 ± 1.8	83.1 ± 0.31	4.6 ± 0.6	2.11	40 ± 3	10.7 ± 0.6	3.70 ± 0.06
	1 mg.	ENT 0.1 mg.	9	280 ± 28	39.5 ± 2.9	85.4 ± 0.16	5.1 ± 0.9	3.52	67 ± 6	8.4 ± 1.2	3.83 ± 0.19
	1 mg.	ENT 1.0 mg.	5	313 ± 16	40.9 ± 1.3	86.8 ± 0.37	4.3 ± 1.3	3.45	76 ± 5	6.0 ± 1.0	3.90 ± 0.07
	5 mg.	0	10	197 ± 15	31.3 ± 1.5	83.9 ± 0.19	7.3 ± 0.5	3.58	56 ± 5	10.7 ± 0.7	3.85 ± 0.21
	5 mg.	P 5 mg.	9	207 ± 11	35.3 ± 2.7	83.4 ± 0.34	5.9 ± 0.3	3.03	58 ± 6	7.7 ± 0.3	4.11 ± 0.16

Fifty μg. ECP controls[b]			8	165 ± 15	29.4 ± 2.2	81.9 ± 0.15	11.9 ± 1.0	—	32 ± 3	8.8 ± 0.7	1.53 ± 0.06
	Placebo		10	148 ± 6	27.3 ± 1.1	81.5 ± 0.15	10.6 ± 1.0	1.00	36 ± 3	9.7 ± 0.9	1.42 ± 0.13
	0	P 5 mg.	10	117 ± 6	21.6 ± 0.8	81.4 ± 0.18	14.2 ± 0.6	1.06	27 ± 2	11.2 ± 1.0	1.83 ± 0.08
	0	ENT 0.1 mg.	8	135 ± 2	25.4 ± 1.4	81.3 ± 0.19	17.3 ± 3.5	1.49	34 ± 1	23.6 ± 4.7	1.66 ± 0.04
	0	ENT 1.0 mg.	5	200 ± 12	35.1 ± 1.6	82.3 ± 0.28	15.5 ± 2.8	1.97	55 ± 5	11.2 ± 1.5	1.85 ± 0.13
Fifty μg. ECP	1 mg.	0	10	186 ± 7	31.5 ± 0.6	82.8 ± 0.19	18.7 ± 1.5	2.21	46 ± 3	11.0 ± 0.7	2.68 ± 0.10
	1 mg.	P 5 mg.	14	255 ± 14	43.0 ± 1.9	83.3 ± 0.25	21.4 ± 1.4	3.48	64 ± 5	15.9 ± 0.8	3.34 ± 0.09
	1 mg.	ENT 0.1 mg.	8	192 ± 9	32.9 ± 2.1	82.6 ± 0.24	17.8 ± 2.1	2.18	53 ± 2	19.1 ± 1.3	2.88 ± 0.07
	1 mg.	ENT 1.0 mg.	6	262 ± 8	44.3 ± 0.7	83.1 ± 0.25	16.2 ± 1.6	2.70	80 ± 3	9.4 ± 0.5	3.75 ± 0.35
	5 mg.	0	8	199 ± 11	34.1 ± 1.8	83.3 ± 0.27	14.6 ± 1.3	1.85	46 ± 3	9.5 ± 0.9	2.88 ± 0.11
	5 mg.	P 5 mg.	8	242 ± 7	41.8 ± 1.2	82.7 ± 0.21	24.4 ± 1.5	3.76	64 ± 5	17.0 ± 1.7	3.79 ± 0.21
	Placebo		5	19 ± 1	3.3 ± 0.07	80.3 ± 0.33	4.3 ± 0.3	—	6 ± 0.4	2.9 (pooled)	1.40 ± 0.03
	0	P 5 mg.	5	27 ± 3	—	—	3.9 ± 0.4	—	4 ± 0.4	3.2 ± 1.3	0.33 ± 0.08
	0	ENT 1.0 mg.	6	77 ± 4	11.7 ± 0.10	84.7 ± 0.21	6.4 ± 0.3	—	14 ± 0.9	7.8 ± 1.0	2.33 ± 0.11
0	1 mg.	0	5	18 ± 0.5	3.0 ± 0.05	81.8 ± 0.32	4.6 ± 0.5	—	5 ± 0.3	4.1 (pooled)	1.95 ± 0.17
	1 mg.	ENT 1.0 mg.	8	98 ± 9	13.8 ± 0.60	85.3 ± 0.29	5.7 ± 0.3	—	20 ± 0.8	6.1 ± 0.3	3.56 ± 0.10
	5 mg.	0	5	34 ± 3	—	—	5.7 ± 0.5	—	9 ± 0.4	5.2 ± 0.3	2.69 ± 0.12
	5 mg.	P 5 mg.	5	42 ± 3	—	—	2.9 ± 0.8	—	8 ± 0.9	3.5 ± 0.1	2.42 ± 0.08

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[a] ECP = estradiol cyclopentylpropionate.

[b] Posted day 8 after single priming dose: that is, at the time other treatments were started.

[c] Injected daily days 8 to 14 after single priming dose day 1. Relaxin was injected in 0.1 ml. 5 per cent beeswax in peanut oil. Progestagens were injected in 0.1 ml. sesame oil.

[d] Standard error of the mean.

[e] P = progesterone.

[f] ENT = 17α-ethyl, 19-nortestosterone.

* 1 mg. relaxin dissolved in saline solution for comparison with repository suspension in beeswax oil.

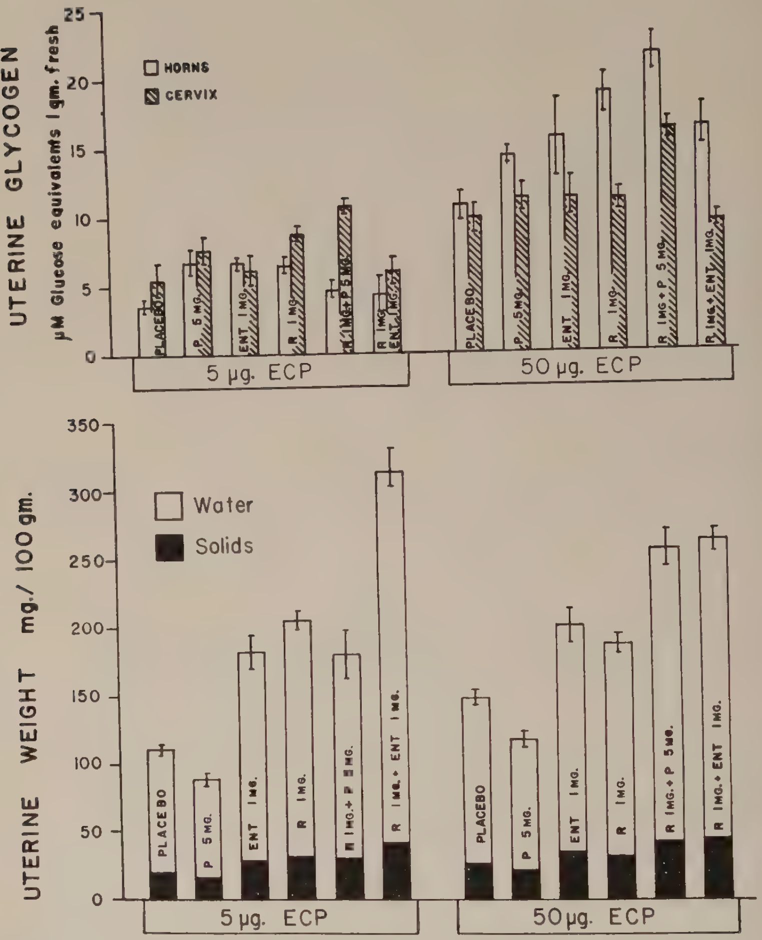

FIGURE 7. Effects of 7 daily injections of relaxin and progestational steroids on uterine weight and composition in the spayed rat primed 1 week previously with 5 or 50 μg. estradiol cyclopentylpropionate. Daily dosages are indicated on the bars. The vertical brackets indicate ± standard error of the mean. Numbers of animals are indicated in TABLE 4.

Effects of progestagens. When 5 mg. dosages of progesterone were injected daily on days 8 to 14 following either priming, there was observed a significant reduction in uterine weight ($p < 0.01$). Water content was not significantly altered (TABLE 4, FIGURE 7). Glycogen concentration increased, but this was roughly proportional to the decrease in uterine weight, the absolute amount of glycogen remaining constant. Progesterone had no appreciable effect on uteri of nonestrogenized animals (TABLE 4), but 17α-ethyl-19-nortestosterone (ENT) was uterotrophic and induced an increase in uterine water in unprimed and primed animals. The effects of this latter hormone were decreased by increas-

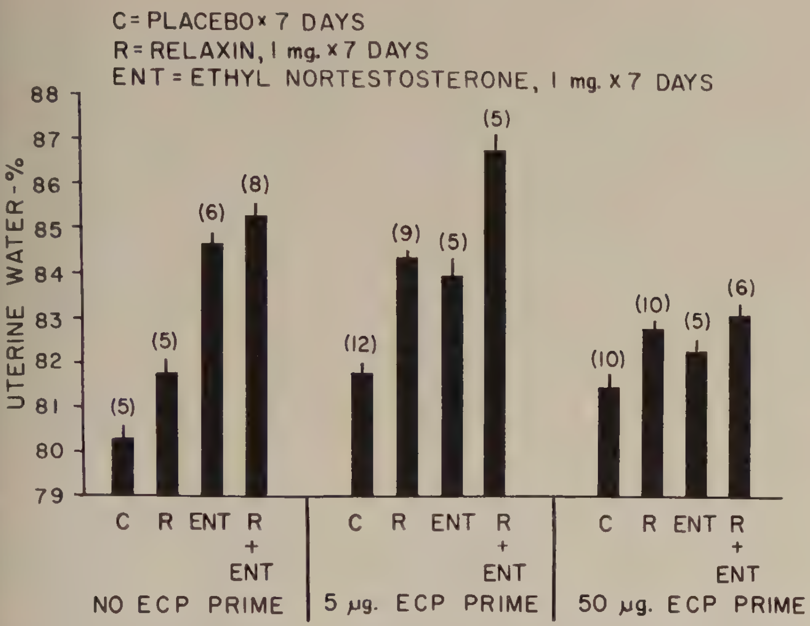

FIGURE 8. Effects of increasing levels of estrogen priming on uterine water response to 7 injections of relaxin and/or 17 α-ethyl-19-nortestosterone. The thin vertical lines indicate 1 standard error; ( ) indicates the number of ovariectomized rats per group.

ing the level of estrogen priming from 5 to 50 μg. ECP (TABLE 4, FIGURES 7 and 8).

Effects of relaxin. Daily injections of relaxin (1 or 5 mg. in beeswax oil) exerted only slight effects on uteri of unprimed rats (TABLE 4). Following priming with 5 μg. ECP, relaxin induced a marked elevation of uterine weight (wet and dry) and water content, and an increase in uterine glycogen concentration (TABLE 4, FIGURE 7). When the 1 mg. daily dosage was given in saline solution, effects on wet and dry weight and water content were slight (TABLE 4). When the estrogen priming dose was increased to 50 μg. ECP, relaxin effects on uterine weight and water content were less marked, although a considerable further increase in uterine glycogen concentration was observed (TABLE 4, FIGURES 7 and 8). All differences were statistically significant at $p < 0.01$.

Effects of relaxin and progestagens. Progesterone, at a daily dosage of 5 mg., did *not* significantly decrease the effects of daily dosages of relaxin (1 or 5 mg.) on uterine weight of rats primed with 5 μg. ECP. When the animals were primed with 50 μg. ECP, progesterone and relaxin synergized. Wet and dry uterine weights were significantly higher ($p < 0.01$) than in all appropriate control groups (TABLE 4, FIGURE 7).

Daily injections of ENT and relaxin were augmentative, particularly following the 5 μg. ECP prime, inducing the greatest increases in uterine weight and water content of any of the treatments employed. Increasing the level of estrogen priming from 5 to 50 μg. ECP decreased the effects of relaxin, ENT, or their combination on uterine water (TABLE 4, FIGURES 7 and 8).

Both progestational compounds appeared to show some synergistic effects with relaxin on uteri of unprimed animals, but the magnitude of the changes was small, and the significance must be questioned until larger series and more dosages are run (TABLE 4).

Factors Influencing Cervical Dilatability

Estrogen priming. Cervices of rats primed with 5 μg. ECP were more dilatable than those of unprimed controls. However, it should be noted that there was a concomitant fourfold increase in cervical weight, and this growth alone may account for the increase in stretchable diameter of the canal. When the estrogen dose was increased to 50 μg., there was observed a decrease in dilatability with a further increase in cervix weight. Thus, this effect was inversely proportional to growth (TABLE 4, FIGURE 9).

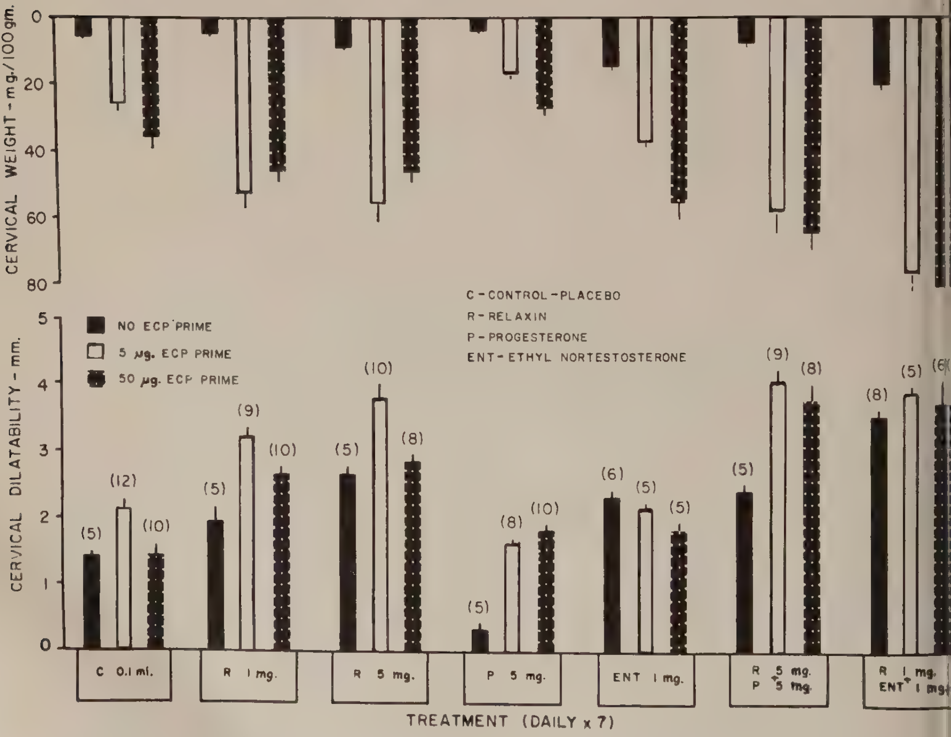

FIGURE 9. Effects of estrogen priming, relaxin, and progestagens on cervical dilatability and weight of the cervical portion of the uterus in nonpregnant ovariectomized rats. The single priming dosage was injected 1 week before commencing daily treatments with relaxin and steroids. The thin vertical lines represent 1 standard error; ( ) indicates the number of rats per group.

Effects of progestagens. Progesterone at a 1 or 5 mg. dosage had no consistent or marked effect on cervical dilatability in primed or unprimed rats. In general, progesterone decreased the size of the cervix and this was accompanied by a decrease in the stretch diameter of the canal (TABLE 4, FIGURE 9).

ENT induced a definite ($p < 0.01$) increase in cervical dilatability of unprimed castrates, but this was accompanied by a twofold increase in weight of the structure. In rats primed with 5 or 50 μg. ECP, ENT induced no change in cervical dilatability that could not be accounted for by increased growth alone; again, however, increasing estrogenization decreased the stretch diameter (TABLE 4, FIGURE 9).

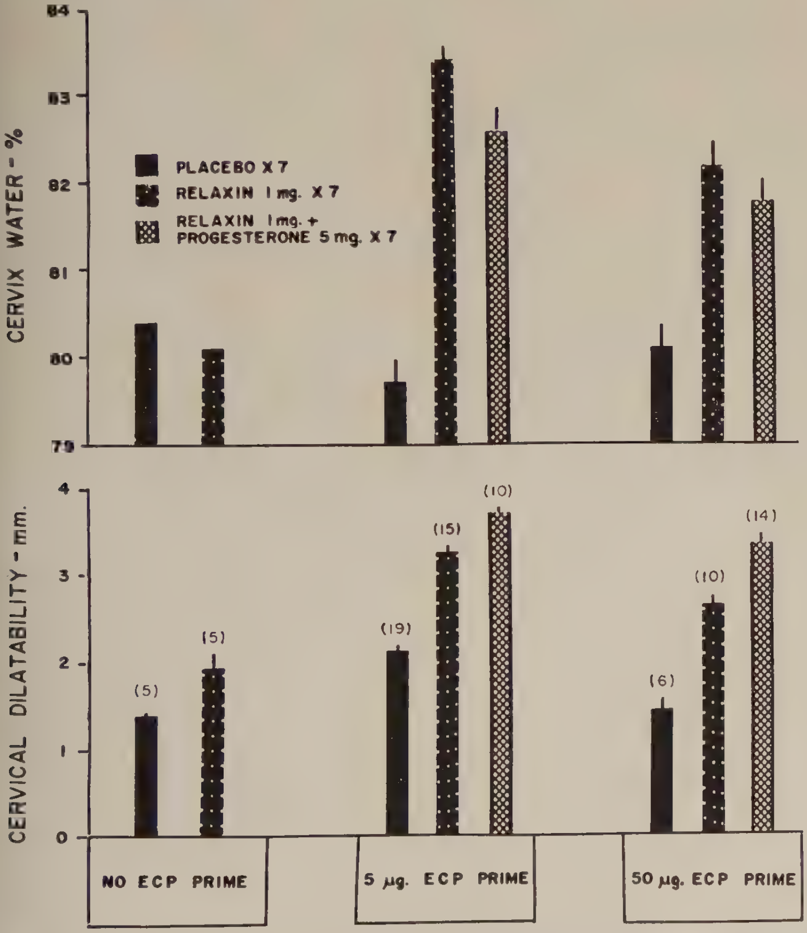

FIGURE 10. Effects of relaxin alone and in combination with progesterone on cervical dilatability and cervical water content in unprimed and estrogen-primed (1 week previously) spayed rats. Note that increased dilatability is not always accompanied by increased water content. Thin vertical lines represent 1 standard error; ( ) indicates the number of rats per group.

Effects of relaxin. Relaxin injected in a beeswax-oil vehicle induced cervical softening in unprimed and primed rats. Again, results are difficult to assess because of the accompanying hypertrophy of the cervix. However, in unprimed rats, 1 or 5 mg. relaxin daily induced dose-proportional increases in dilatability without increasing cervical weight markedly. In rats primed with 5 μg. ECP, there was observed increased cervical softening with increasing relaxin dosage from 1 to 5 mg. daily in beeswax oil, although cervical weights were similar in the 2 groups (TABLE 4, FIGURE 9). A daily dosage of 1 mg. relaxin in saline solution did *not* induce cervical softening (see TABLE 4).

Cervices of relaxin- (in wax oil) treated rats were significantly less dilatable ($p < 0.01$) following the 50 μg. ECP prime than when the 5 μg. estrogenization was employed (FIGURE 9).

Effects of progestagens and relaxin. Relaxin (1 mg.) and progesterone (5 mg.) synergized to increase cervical dilatability significantly ($p < 0.01$) in rats primed with 5 μg. ECP, although there was no increase in the weight of the cervical portion. Following a 50 μg. ECP prime, relaxin and progesterone treatment induced both increased dilatability and cervical hypertrophy (TABLE 4).

Similar synergism was observed when combinations of relaxin and ENT were injected into unprimed or primed rats. This was invariably accompanied by cervical hypertrophy (TABLE 4, FIGURE 9).

Cervical water as a factor in cervical softening. Increasing estrogenization partially prevented the water uptake of the cervix due to daily relaxin treatment, and it appeared that the decrease in dilatability might be accomplished by this mechanism. However, concomitant treatment with relaxin and progesterone reversed the estrogen effect on dilatability, whereas cervical water content was unchanged or slightly reduced (TABLE 4, FIGURE 10).

Effects of Multiple Injections of Relaxin and Progesterone in Estrogen-Primed Ovariectomized Mice: Preliminary Study

Four groups of spayed mice were estrogen primed with 5 μg. ECP. One week later daily injections of placebo, 0.1 mg. relaxin in 0.2 ml. L-390, 1 mg. progesterone in 0.1 ml. sesame oil, or a combination of relaxin and progesterone at the above dosages were begun. Under these conditions, relaxin induced a small but significant increase in uterine weight, a two- to threefold increase in uterine and cervical glycogen concentration, a tenfold increase in the length of the interpubic ligament, and a 1-mm. increase in cervical dilatability without increasing cervical weight (FIGURE 11). Progesterone reduced uterine and cervical weight very markedly, but cervical dilatability was nevertheless increased. Progesterone also effected a definite decrease in uterine glycogen concentration. When relaxin and progesterone were injected concomitantly for seven days, uterine and cervical weight, glycogen concentration, and interpubic ligament length were significantly reduced when compared with values obtained in animals treated only with relaxin. Cervical dilatability was not affected (FIGURE 11). It is not as yet known whether these relationships

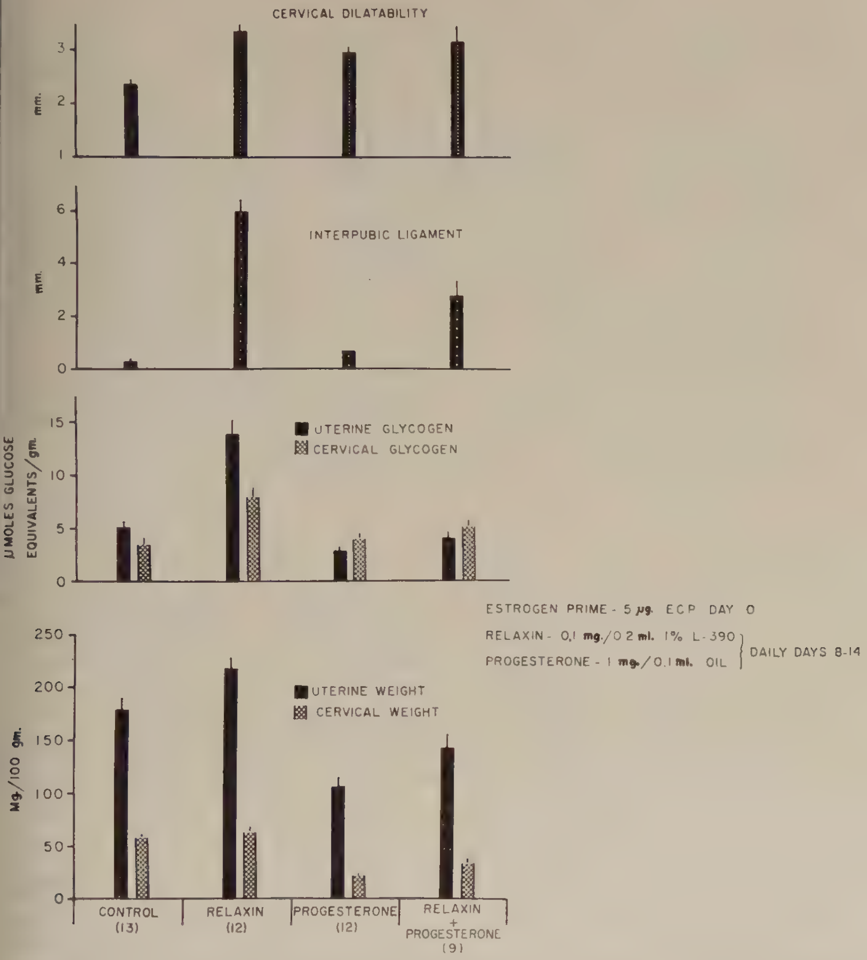

FIGURE 11. Effects of 7 daily injections of relaxin and progesterone on the uterus, cervix, and symphysis pubis of ovariectomized mice primed 1 week previously with 5 µg. estradiol cyclopentylpropionate. The thin vertical lines represent 1 standard error; ( ) indicates the number of mice.

would hold when higher dosages of relaxin were given, and the data therefore are not comparable to those obtained for rats.

Effects of Estrogen, Progesterone and Relaxin in Ovariectomized Pregnant Rats

Criteria of successful pregnancy maintenance in rats spayed on the fifteenth day and sacrificed on the twenty first day of gestation included (1) number and condition of fetuses relative to implantation sites; (2) normal uterine and placental glycogen concentration; (3) normal uterine water content; and (4) normal cervical water content and dilatability.

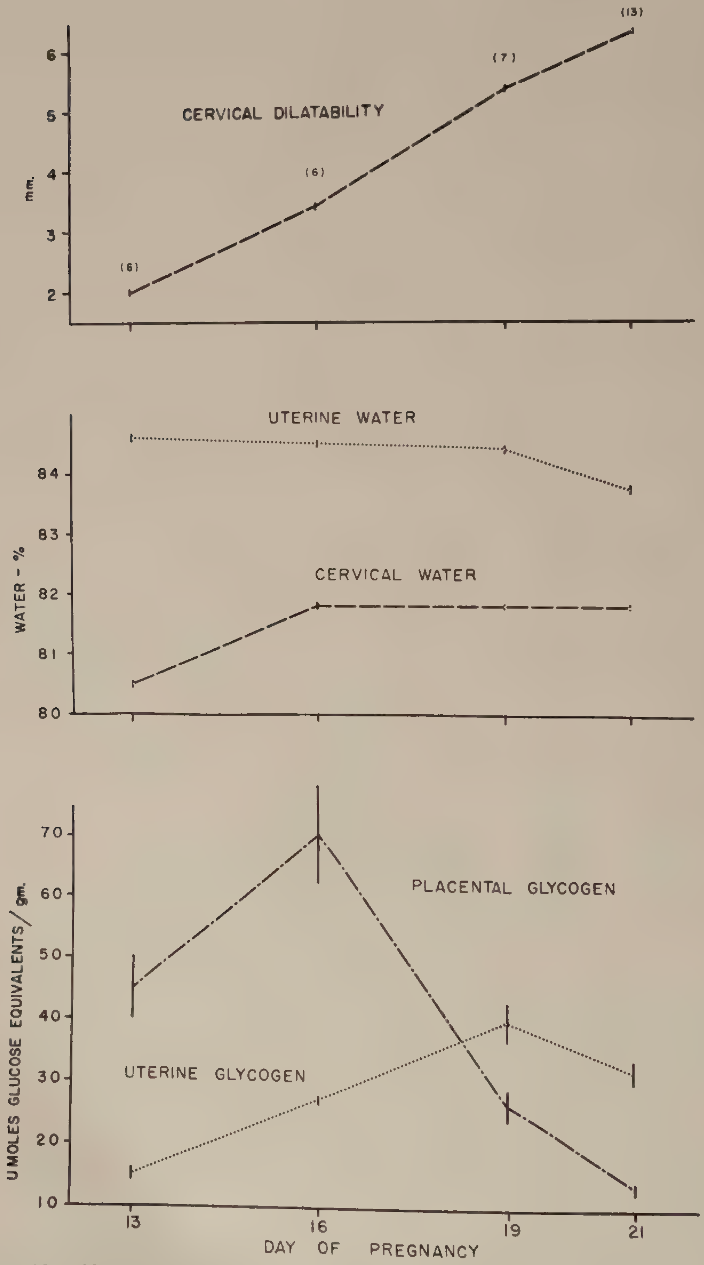

FIGURE 12. Changes in glycogen concentration of the uterus and placenta, water content of uterus and cervix, and dilatability of the cervix with advancing pregnancy in intact rats The vertical lines represent standard errors of the means; ( ) indicates the number of rats sacrificed at each time interval.

Intact untreated pregnant rats were sacrificed on the thirteenth, sixteenth, nineteenth and twenty-first days of gestation in order to characterize normal values and direction of changes (FIGURE 12). Uterine glycogen was observed to increase from the thirteenth to nineteenth day, whereas placental glycogen reached a maximum concentration on the sixteenth day and then dropped to one sixth of this value on the twenty-first day of pregnancy. Uterine water levels remained elevated until the nineteenth day, and then decreased slightly. Cervical dilatability was observed to increase steadily from the thirteenth to twenty-first day, confirming the reports of DeVaal[38] and Harkness and Harkness.[39] Only the dilatability increase occurring between the thirteenth and sixteenth day was correlated with an increase in cervical water content. Maximum fetal wastage (that is, number of implantations not represented by living fetuses) was approximately 12 per cent in rats sacrificed on day 21 of pregnancy. No damaged living fetuses were found in this series.

Spayed rats were treated with 0.5, 1.0, 2.0, 5.0, or 10 mg. progesterone daily from the day of ovariectomy (day 15 of pregnancy). If maintenance at a given

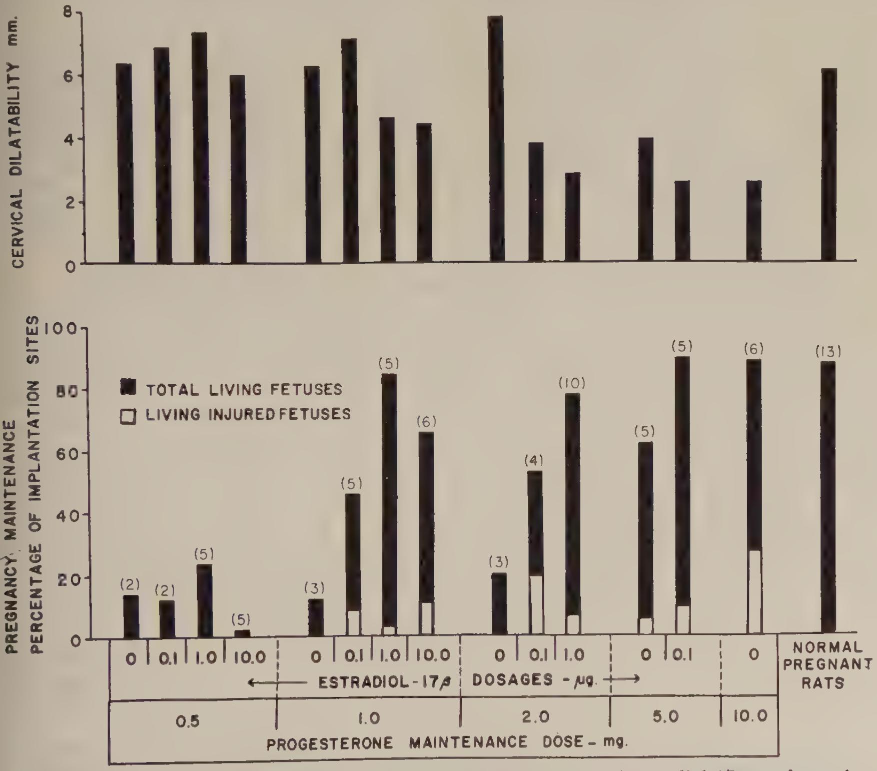

FIGURE 13. Effects of graded daily dosages of progesterone and estradiol-17β on the maintenance of fetuses in rats ovariectomized on the fifteenth day of gestation, and the inverse relationship between pregnancy maintenance and cervical dilatability on the twenty-first day. All dosages indicated were injected daily from the fifteenth through the twentieth day; ( ) indicates the number of rats.

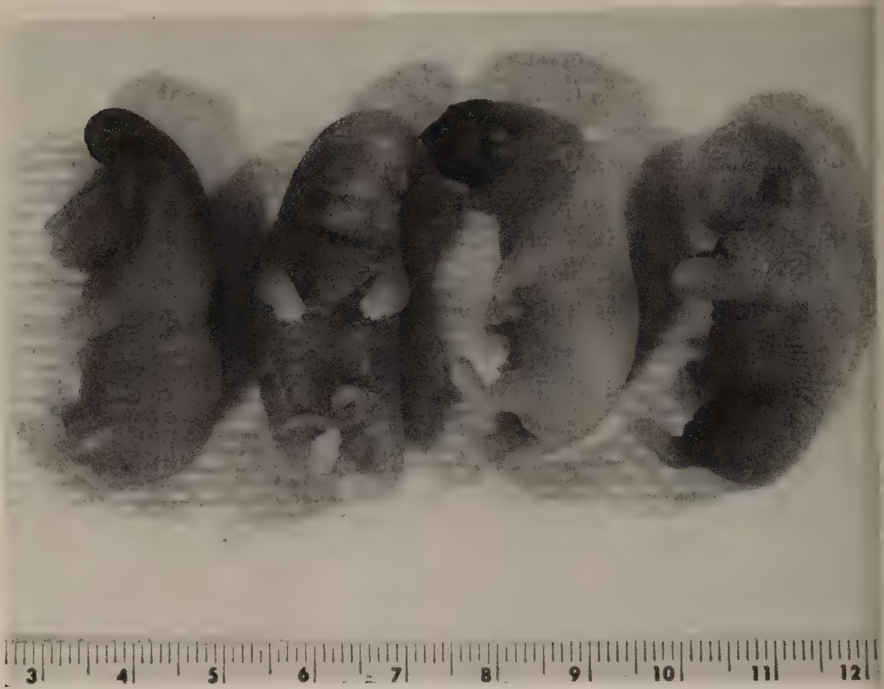

FIGURE 14. Damaged fetuses removed from uteri of spayed pregnant rats maintained on daily injections of 10 mg. progesterone. Ovariectomy: day 15. Sacrifice: day 21. Deformities and hematomata generally were observed at head and tail regions. Fetuses were usually alive despite extensive injury.

dose was generally unsuccessful, daily injections of estradiol-17β were given concomitantly in dosages of 0.1, 1.0, or 10.0 μg. to establish possible maintenance synergism. Under these conditions, it was found that if progesterone were the only therapy a daily dosage of 10 mg. was necessary to maintain normal litter size (FIGURE 13). However, a large proportion of fetuses was deformed and injured (FIGURE 14). Similar observations have been made independently by Alexander *et al.*[40] and Pitel (personal communication). The successful progesterone maintenance dose could be reduced to 1 or 2 mg. daily by concomitant injection of 1 μg. (but not 0.1 μg.) estradiol-17β, or to 5 mg. by concomitant injection of 0.1 μg. estradiol-17β. Under these conditions injury to fetuses was reduced.

However, it was observed that increased maintenance varied inversely with dilatability of the uterine cervix. Thus, spayed rats in which pregnancy was successfully maintained showed less dilatable cervices than intact animals sacrificed on the same day of gestation (FIGURE 13). It cannot be stated with certainty whether the increased dilatability observed in animals failing to hold their litters was related to the cause or to the result of abortion.

Guinea pig bioassay of ovaries removed from rats on the twenty-first day of

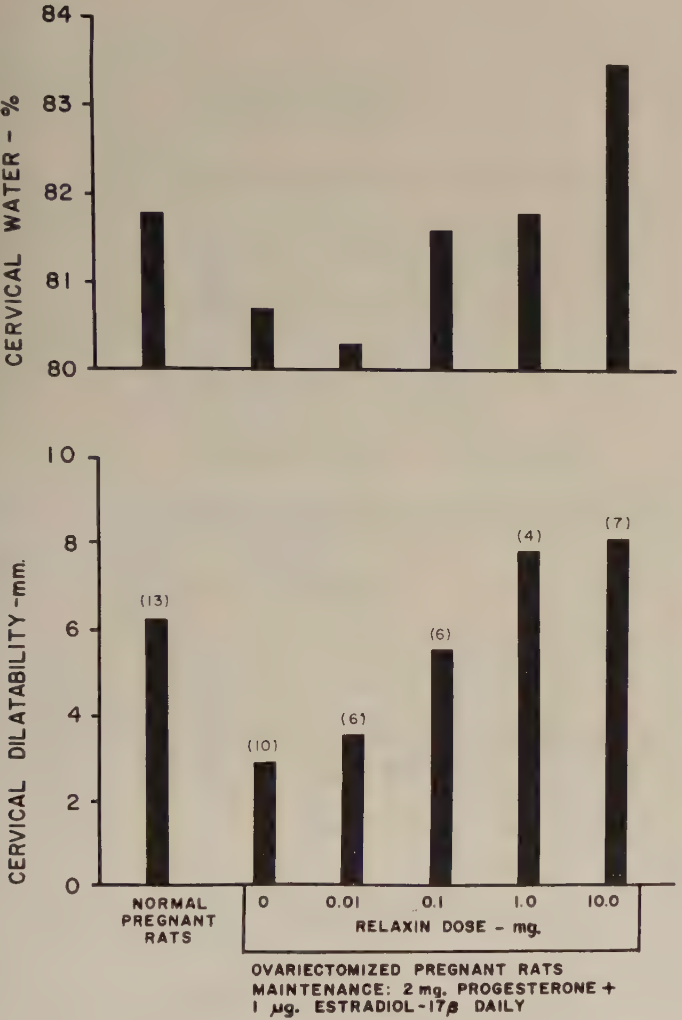

FIGURE 15. Effects of relaxin on cervical dilatability and water content in spayed pregnant rats maintained on progesterone and estradiol-17 β. Relaxin daily dosage, as indicated, was injected in 5 per cent beeswax in peanut oil. Ovariectomy: day 15. Sacrifice: day 21 of pregnancy.

pregnancy indicated an activity equivalent to approximately 4 to 5 mg. (600 to 750 g.p.u.) of relaxin standard per gram fresh. No activity was detected in placentae (unpublished data).

Accordingly, spayed rats maintained on 2 mg. progesterone and 1 μg. estradiol-17β daily were treated concomitantly with relaxin. Dosages of 0.1 or 1.0 mg. restored cervical dilatability and cervical water content to essentially normal values without significantly modifying pregnancy maintenance (FIGURE 15).

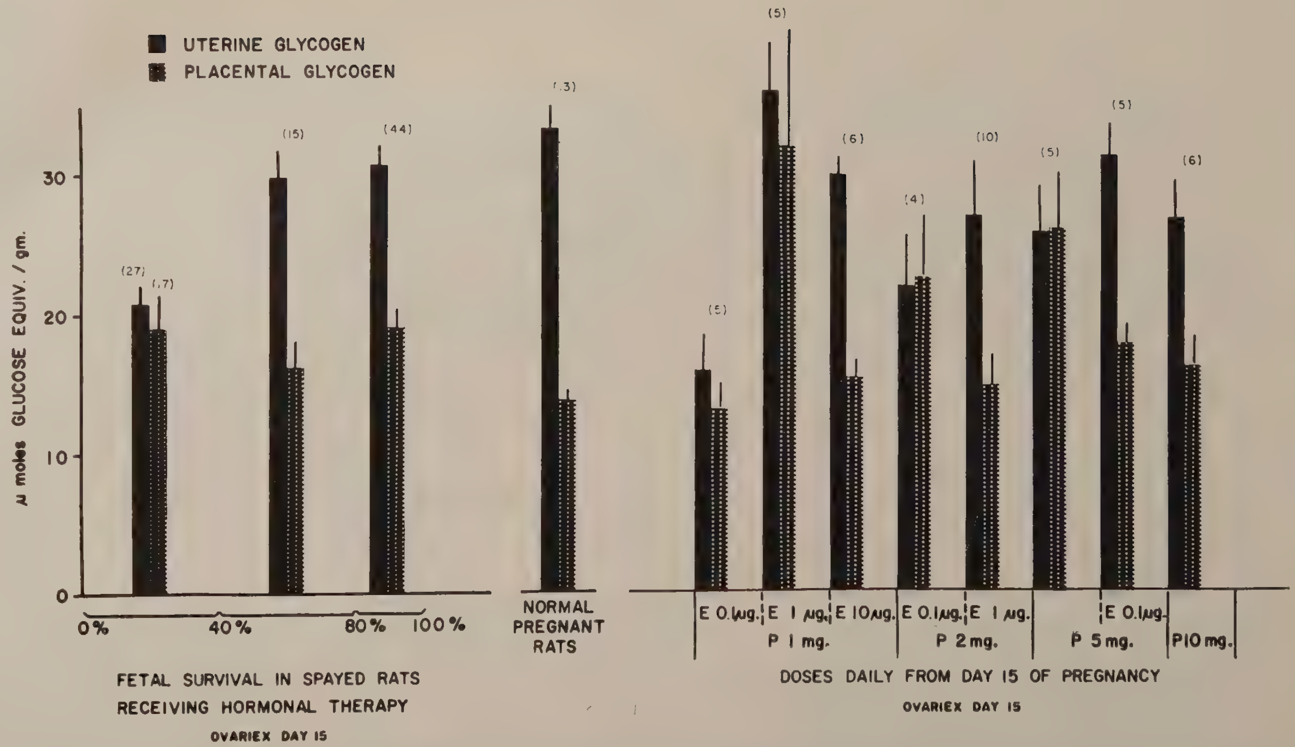

FIGURE 16. Factors influencing uterine and placental glycogen concentration in spayed pregnant rats maintained on various combinations of progesterone and estradiol-17β. On the the left side of the graph data were plotted on the basis of fetal survival intervals without regard for specific hormonal therapy. On the right side, the data were plotted according to treatment (compare with pregnancy maintenance data on the same treatments in FIGURE 13); ( ) indicates the number of rats. Uterine glycogen levels were lower than normal when fewer than 50 per cent of the fetuses were carried to the twenty-first day.

A daily dosage of 10 mg. relaxin did not interfere with pregnancy maintenance, although cervices of some individuals had greater stretch diameters than the instrument was capable of recording (that is >10 mm.). This would at least suggest that in the rat a fully dilatable cervix is not necessarily a predisposition to abortion.

An attempt was made to ascertain the factors responsible for the extremely high glycogen concentration in the pregnant uterus. The data were calculated both as to specific hormonal therapy and to fetal survival (disregarding therapy). It appeared that uterine glycogen was not primarily dependent upon any specific estrogen-progesterone levels, but only upon fetal maintenance (FIGURE 16—compare with FIGURE 13). Thus, glycogen levels were significantly low when maintenance was less than 40 per cent, or when insufficient estrogen-progesterone therapy was administered following ovariectomy. Surprisingly, placental glycogen concentration was apparently normal or high even when fetal maintenance was poor. The highest placental glycogen values were observed in animals treated with 1 mg. progesterone and 1 μg. estradiol 17β daily, but the series is too small and the standard error too large to allow us to attach any particular significance to this finding.

Effects of Estrogen, Progesterone and Relaxin on Spontaneous Delivery in Spayed Rats

In order to assess the possible importance of the previously mentioned findings on function of the reproductive tract during parturition, the following experiments were performed.

Spayed (day 15) rats were maintained on 2 mg. progesterone and 1 μg. estradiol daily until the nineteenth day of pregnancy. Progesterone was either discontinued, or one additional dosage of 1 mg. was given on day 20. Estradiol injections were continued until day 22. Under these conditions, some rats were observed to deliver a few living young; in others, dead young or no young were found in cages. When these animals were sacrificed on day 23 (1 day after expected parturition) retained placentae were invariably found. In addition, many animals still retained a few living or dead fetuses (TABLE 5).

Concomitantly run animals were treated as above, but in addition, 1 mg. relaxin was injected daily. Only 1 rat of 15 evidenced retained placentae and fetuses. The other 14 delivered their entire litters prior to the twenty third day (TABLE 5).

Measurements of cervical dilatability in both groups offer no clue as to the mechanism of retained placenta (TABLE 5), as there was no difference observed between the 2 treatments. The data do suggest again that increased cervical dilatability can be induced either by withdrawal of estrogen and progesterone or by addition of relaxin therapy.

Effects of Estrogen, Progesterone and Relaxin in Mice Ovariectomized the Sixteenth Day of Pregnancy

When mice were spayed on the sixteenth day of pregnancy, abortion occurred in 24 to 30 hours unless progesterone therapy (1 mg. daily) was instituted at the time of operation (TABLE 6).

TABLE 5

EFFECT OF RELAXIN ON SPONTANEOUS PARTURITION IN SPAYED (DAY 15) PREGNANT RATS MAINTAINED ON PROGESTERONE AND ESTRADIOL ANIMALS SACRIFICED 23 DAYS POSTMATING

Treatment (daily)	N	Percentage of mothers with retained placentae	Average No. placentae per mother	Average No. fetuses *in utero* per mother		Births observed per mother		Cervix dil. mm.
				Living	Dead	Living	Dead	
Progesterone, 2 mg. daily days 15–19; estradiol 17β, 1 μg. daily days 15–22	3	100	7.7 ± 0.7	0.33 ± 0.33	1.7 ± 0.9	0	1.0 ± 0.6	8.6 ± 1.4
As above, except 1 mg. progesterone day 20	10	100	6.7 ± 1.0	2.0 ± 1.3	1.4 ± 0.7	0.8 ± 0.4	1.3 ± 0.5	6.4 ± 0.8
Progesterone, 2 mg. daily days 15–19; estradiol 17β, 1 μg. daily days 15–22; relaxin, 1 mg. daily days 15–22	4	0	0	0	0	1.2 ± 0.9	6.7 ± 2.5	7.2 ± 0.3
As above, except 1 mg. progesterone day 20	11	9	0.7 ± 0.7	0	0.09 ± 0.09	1.7 ± 1.0	1.3 ± 0.8	8.4 ± 0.2
Combined data:								
Progesterone and estradiol only	13	100	7.0 ± 0.9	1.6 ± 1.0	1.5 ± 0.5	0.6 ± 0.4	1.2 ± 0.4	6.9 ± 0.7
Progesterone, estradiol, and relaxin	15	7	0.5 ± 0.5	0	0.07 ± 0.07	1.6 ± 0.7	2.7 ± 1.0	8.0 ± 0.3

TABLE 6
INFLUENCE OF PROGESTERONE AND RELAXIN ON TIME OF PARTURITION IN SPAYED (DAY 16) PREGNANT MICE

Treatment	No. mice	Percentage of mice pregnant on day:			
		19	20	21	22
None	9	0 (All aborted 24 hrs. post op.)			
Relaxin (2.0–6.7 mg. in saline or beeswax oil), day 16–17	8	0 (All aborted 24 hrs. post op.)			
Progesterone, 1 mg. daily from day 16	8	100	100	100	100
Progesterone, 1 mg. daily from day 16 + relaxin, 6.7 mg., days 16, 18, 20	8	100	75	50	50
Progesterone, 1 mg. days 16–19	7	100	100	71	57
Progesterone, 1 mg. days 16–19 + relaxin, 6.7 mg., days 16, 18	7	100	57	0	—

Pregnancy was prolonged when progesterone injections were continued through the twenty-first day, and fetal mortality was approximately 68 per cent on the twenty-second day. Concomitant relaxin therapy did not significantly alter this effect. When progesterone injections were discontinued the nineteenth day, results were variable. Some mice delivered living and dead young by the morning of the twenty-first or twenty-second day. In others, pregnancy was prolonged and the mothers exhibited extremely toxic symptoms, two of them being moribund on the twenty-second day. Fetal mortality in utero was 96 per cent. When a concomitantly run group of mice was pretreated with relaxin, uterine evacuation was in all cases completed 24 to 48 hours after cessation of progesterone treatment (TABLE 6). Some viable young were found in the cages.

In order to achieve better control of such experiments, oxytocin induction of delivery was attempted on the nineteenth day of gestation. Spayed animals were compared with intact and unilaterally ovariectomized mice. All mice were individually caged and closely observed for 4 hours following a single injection of 0.5 I.U. oxytocin in 20 per cent PVP. Results of induction were expressed as the average of the percentages of each litter delivered.

Unilaterally ovariectomized mice were significantly ($p < 0.01$) more responsive to oxytocin than intact or sham-operated mice. Bilaterally ovariectomized mice maintained on 1 mg. progesterone daily were generally resistant to oxytocin injection. Progesterone also significantly reduced responsiveness to oxytocin in intact and unilaterally ovariectomized mice ($p < 0.01$). Relaxin (6.7 mg. days 16 and 18), but not estradiol 17β (at a variety of dosage levels), reversed the effects of progesterone in ovariectomized mice and partially restored responsiveness of the delivery mechanism in intact mice (FIGURES 17 and 18). A detailed account of these experiments has been published elsewhere.[8]

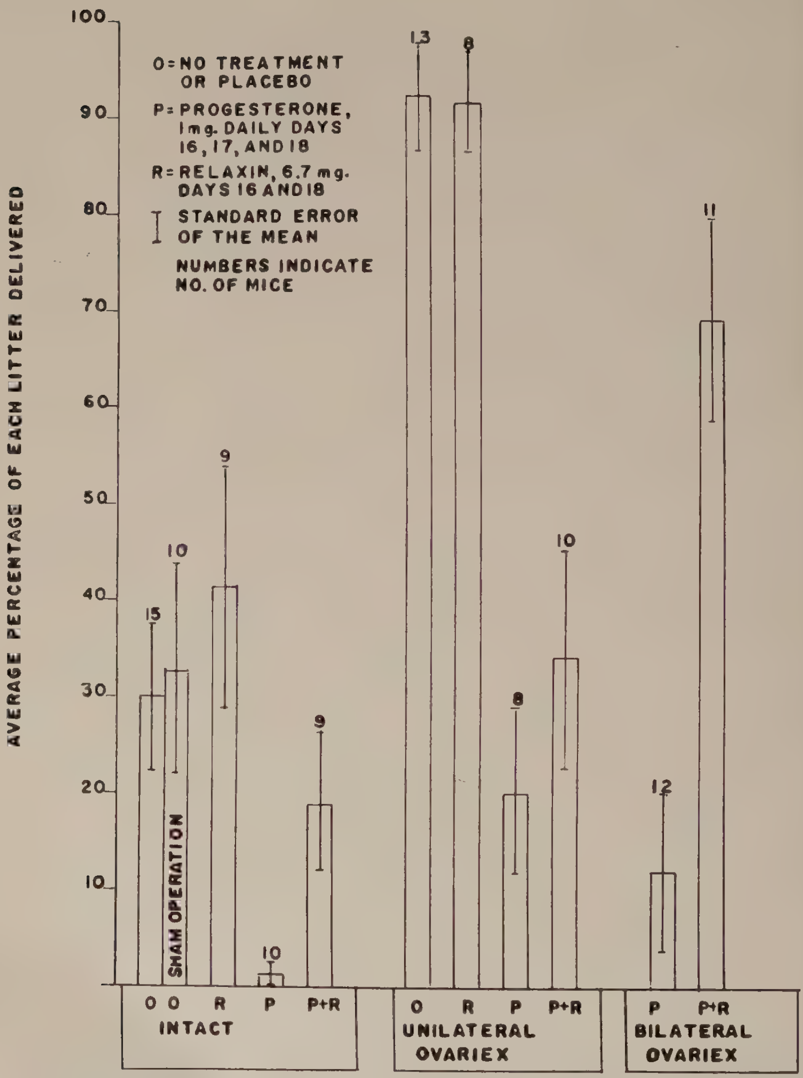

FIGURE 17. Effects of unilateral and bilateral ovariectomy, progesterone, and relaxin on delivery response to oxytocin injection on the ninteenth day of pregnancy in mice. Reproduced by permission from *Endocrinology*, volume 61, page 276, 1957.

Effects of Relaxin on Prolonged Pregnancy Due to Progesterone Treatment of Intact Mice

Seven groups of pregnant mice were treated with relaxin, progesterone (at three dosage levels), or their combination daily from the sixteenth day until the twenty-third day of pregnancy unless parturition intervened (FIGURE 19).

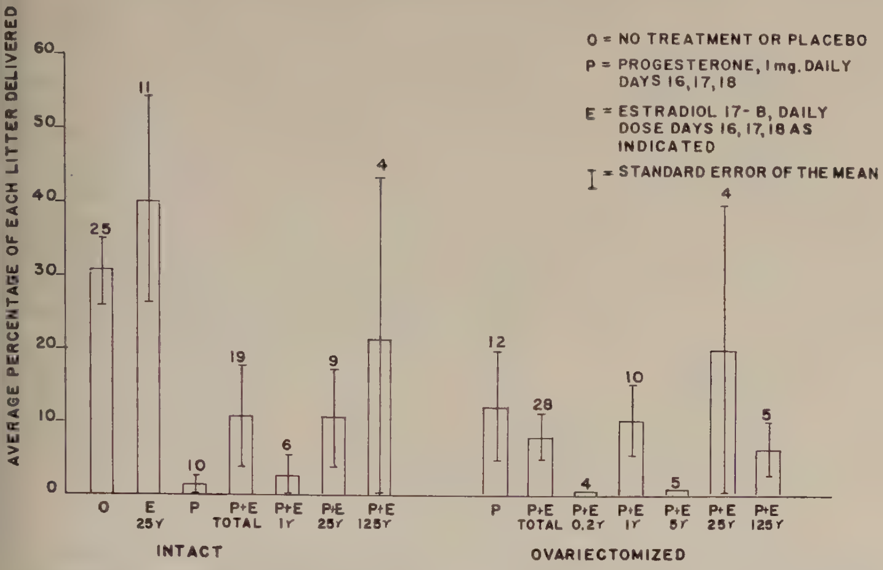

FIGURE 18. Lack of influence of estradiol 17β on response to oxytocin on the nineteenth day of pregnancy in intact and ovariectomized mice treated with progesterone. The numbers indicate the number of mice per group. Reproduced by permission from *Endocrinology*, volume 61, page 277, 1957.

Untreated control and relaxin-treated mice delivered their litters uneventfully between the mornings of the twentieth and twenty-second day, the majority of births occurring before the morning of the twenty-first day.

A daily dosage of 1 mg. progesterone prolonged pregnancy in only a few animals of a group so treated. No fetal deaths were recorded, in contrast to effects observed in spayed mice treated with the identical progesterone dosage. Daily injection of 2.5 mg. progesterone prolonged pregnancy in 100 per cent of mice so treated until sacrifice on the twenty-fourth day. It should be noted that this is higher than the dosage necessary to prolong pregnancy in spayed mice. A daily dosage of 10 mg. progesterone was toxic to mother and fetuses and the experiment had to be terminated on the twenty-third day, at which time all mice still retained litters. Most of the young were dead. Combination of relaxin with any of the above progesterone dosages did not modify results (FIGURE 19).

Pregnancy was also prolonged 2 to 3 days by a single injection of 10 mg. progesterone in a repository vehicle on the nineteenth day. Again, concomitant relaxin injection (in beeswax oil) did not prevent the effect (FIGURE 20).

Similar series of mice were tested for delivery response to oxytocin during prolonged pregnancy. A significant difference was observed between groups on the twenty-third day of gestation. Eleven mice which received relaxin in addition to progesterone were induced to deliver 43 young (an average of 45 per cent of each litter) of which 21 were viable. Twelve mice that received

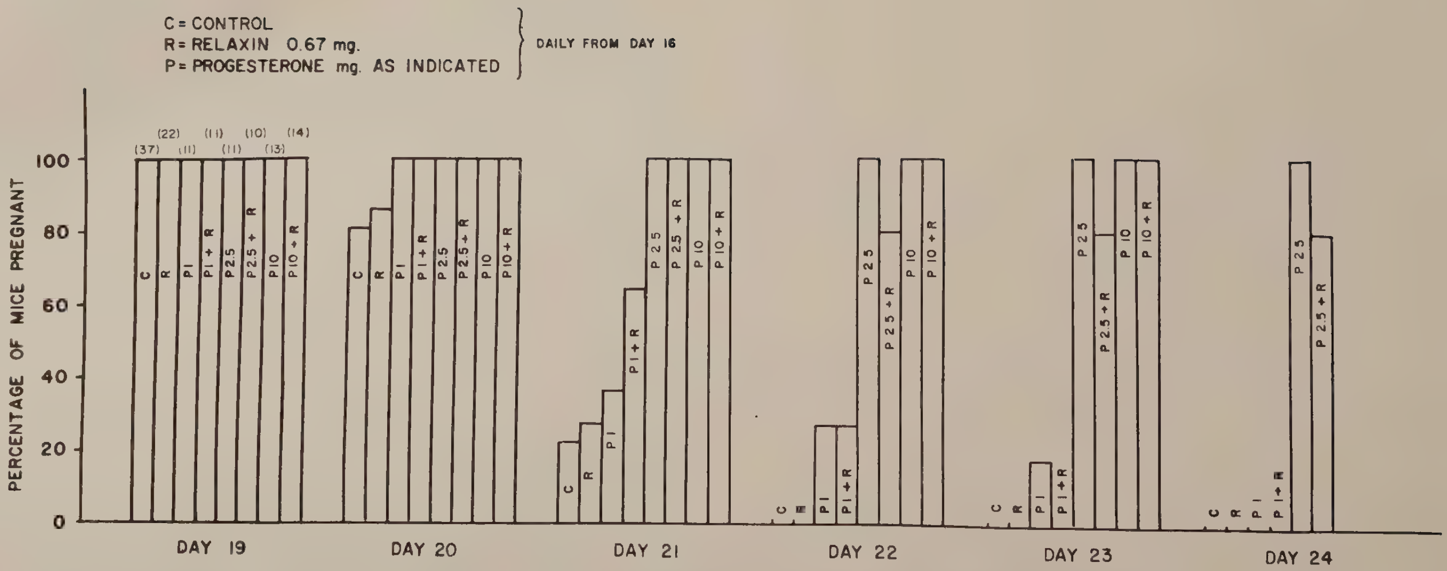

FIGURE 19. Influence of progesterone and relaxin on the time of parturition in intact mice; ( ) indicates the number of mice on each experimental treatment. Experiments, in which a daily dosage of 10 mg. progesterone was injected (daily from day 16), were terminated on the twenty-third day of gestation because of poor condition of the animals. Relaxin was injected in 0.1 ml. 5 per cent beeswax in peanut oil.

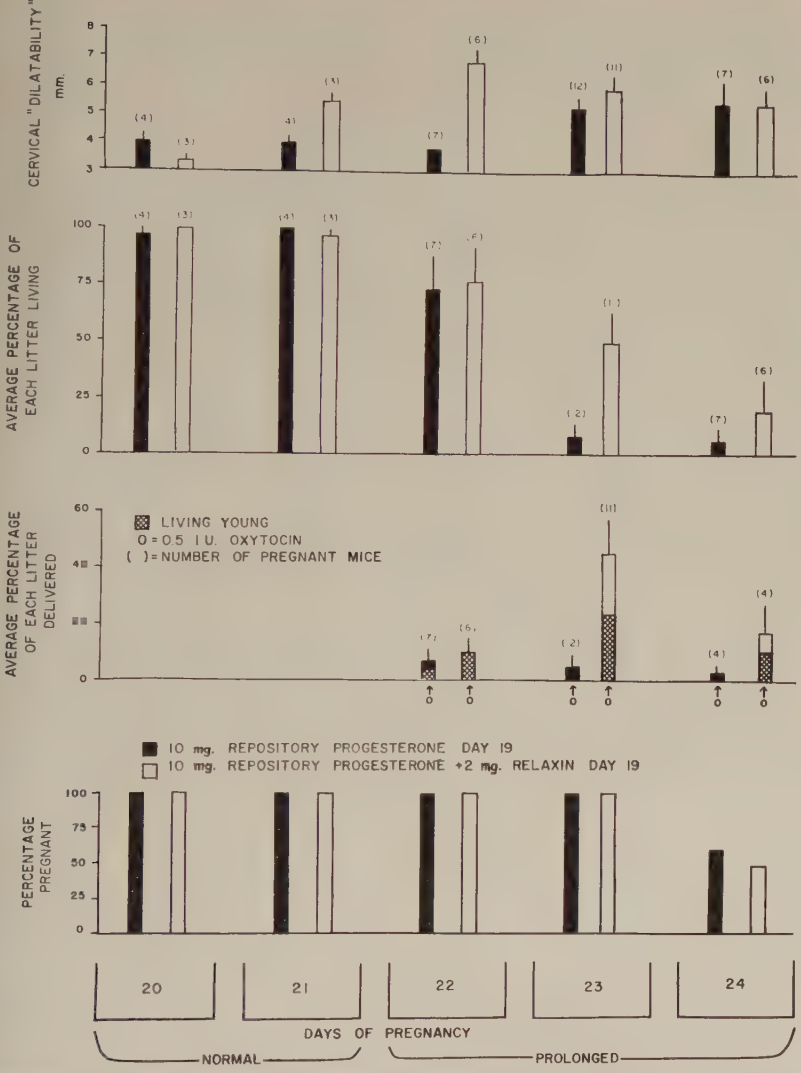

FIGURE 20. Effects of relaxin on oxytocin response, fetal survival, and cervical dilatability in progesterone-prolonged pregnancy in intact mice. The thin vertical lines represent 1 standard error.

progesterone alone delivered a total of 3 dead young (an average of 5 per cent of each litter, FIGURE 20).

When total young (in utero and delivered) were examined, it was found that only 8 per cent of each litter was viable in the group treated with progesterone alone, whereas an average of 50 per cent of each litter survived in the group which received the relaxin in addition (FIGURE 20).

Cervical dilatability measurements did not reveal any difference between groups on the twenty-third day, and they suggested that other factors may have been responsible for the differences in oxytocin responsiveness of the de-delivery mechanism (FIGURE 20).

DISCUSSION

These and other recent studies suggest that a variety of hormones influences the structure and function of the reproductive tract, and that some effects generally attributed to the ovarian steroids may in fact be due to the action of relaxin or a relaxinlike nonsteroidal substance or substances.

Factors affecting uterine growth and composition. True growth of the uterus (increased nitrogen) is generally preceded by an increase in uterine water content.[41, 42] When estrogenic substances were used to promote growth, the water increase was transient,[41-43] whereas relaxin or ethylnortestosterone maintained high uterine water levels as long as therapy was continued.[i] There appeared to be a marked difference in the mode of action because continued or elevated estrogen levels prevented the expected increase in uterine water due to relaxin or ethylnortestosterone. This inhibition of uterine water increase by estrogen itself may explain the seeming paradox of Astwood's[44] finding that estrogen injection induced a rapid rise in uterine water in all stages of the estrous cycle in rats—except estrus itself.

True growth of the atrophied uterus (increased dry weight and nitrogen) was observed in spayed rats treated with estrogen or ethylnortestosterone; only slight growth was recorded when high dosages of relaxin or progesterone were injected repeatedly. Zarrow[30] observed no true growth of the uterus in immature rats treated only with relaxin. Following a priming with a small dose of estradiol cyclopentylpropionate, the uterus responded to relaxin therapy with a marked rise in uterine weight (wet and dry) that could be maintained by continued treatment.

The two progestagens employed had opposite effects on the uterus of estrogen-primed rats: progesterone treatment reduced uterine weight following a small or large ECP priming dose, whereas ethylnortestosterone further increased uterine weight in animals previously primed. It should be noted that this effect is opposite to that reported when ethylnortestosterone and estrogen were given concomitantly.[45]

When progesterone and relaxin were administered to animals receiving a large priming dose of estrogen, uterine weight was significantly greater than those observed in all factorial controls, and uterine composition was similar to that of rats in late pregnancy. This finding strongly suggests a synergism of the various factors of the ovarian complex.

A combination of relaxin and ethylnortestosterone also induced augmented uterine growth in primed rats, but this could be interpreted as an additive effect, since both hormones were uterotrophic.

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[i] Jablonski and Velardo[59] did not observe maintenance of elevated uterine water levels with continued relaxin therapy. Their negative finding is probably due to lack of use of a repository vehicle. We have obtained only slight uterotrophic effects with relaxin in saline solution (TABLE 4).

Uterine glycogen was observed to increase under a variety of experimental conditions, but the effects appeared to be estrogen-limited. Thus, relaxin increased glycogen concentration only 75 to 100 per cent, regardless of dose, in rats receiving the smaller estrogen priming. When the level of priming was raised, relaxin treatment induced a further increase in glycogen concentration.

It was observed that glycogen concentration gave no indication of glycogen deposition. When glycogen deposition paralleled uterine growth, there was no change in glycogen concentration, although glycogen per uterus was vastly increased. Conversely, as uterine weight decreased in primed rats treated with progesterone, glycogen per uterus remained constant, giving a net *increase* in *concentration.* Glycogen increases in uteri of rats treated with relaxin or ethylnortestosterone were often masked by a 2 to 4 per cent increase in uterine water.

Factors affecting cervical dilatability. Measurements of cervical dilatability were complicated by the observation that an increase in the size of the cervical portion of the uterus generally accompanied the various treatments employed. There was a distinct impression that an increase in stretchable diameter was often due simply to an increase in available tissue and not to softening per se. The measurement technique was subjective and, although reproducible results were obtained uniformly in estrogen-primed animals, the very small cervices of unprimed spayed rats were difficult to evaluate, and considerable error was likely.

Estrogen was observed to increase dilatability over castrate values, but this was accompanied by a fourfold increase in weight of the cervical portion. When the estrogen level was raised, there was further increase in cervix weight, but dilatability was significantly decreased. This is in agreement with the data of Smith and Nalbandov,[46] who found that estrogen treatment or natural estrus in sows reduced dilatability of the cervix.

Relaxin treatment was observed to increase dilatability of the cervix proportionately to dosage in unprimed and primed rats. As cervical weight was never increased more than twofold over control values, this is interpreted as a true softening effect, particularly since it could be prevented by high dosages of estrogen. Relaxin-induced cervical softening has been reported previously by many investigators[22-27] who apparently did not take cervical size into account.

Progesterone had little or no effect on rat cervix dilatability that could not be related to changes in cervical size. However, an increased dilatability accompanied by a decreased cervical weight was observed in spayed, estrogen-primed mice treated with progesterone. This finding may be relevant to the previous observation[8] that cervical dilatability was normal in near-term spayed pregnant mice maintained on progesterone alone.

Combined treatment of primed or unprimed rats with relaxin and progesterone or ethylnortestosterone increased cervical dilatability markedly over all factorial control values. The synergistic effect of progesterone and relaxin was more striking because cervical weight was not increased further.

Cullen and Harkness[24] have recently reported similar results using an objective technique to measure cervical stretch.

The mechanism for the increase in dilatability following treatment with relaxin or relaxin and progesterone is unknown. Zarrow[30] found a correlation between greater dilatability, increased water content, and changes in histochemically demonstrated mucopolysaccharides, which he interpreted as depolymerization. Present observations do not exclude these possibilities, but under certain conditions dilatability was increased significantly without a concomitant increase in cervical water concentration. Perhaps the observations of Harkness and Harkness[47] on the significant increase in collagen content of the rat cervix with advancing pregnancy offer a promising lead as to the mechanism of action of these hormones.

Factors influencing pregnancy maintenance. Evaluation of replacement therapies following ovariectomy in pregnant animals is complicated by the continued presence of the placenta as a potential source of similar hormones. A high level of relaxin activity was found only in ovaries of pregnant rats and mice, and not in placentae or uteri. Bloom *et al.*,[48] using an *in vitro* rat uterine segment assay, found a uterine relaxing factor only in relaxin-type extracts of pregnant rat ovaries, and not in extracts of placentae or excised metrial glands. These findings suggest, but do not prove, that the ovary is the important source of relaxin in pregnant mice and rats. Nothing is known of the relative concentrations of estrogens and progestagens in these pregnant rat or mouse tissues.

Maintenance of pregnancy (ideally a full complement of fetuses) in the spayed rat or mouse required progestational hormone therapy.[8, 12, 19, 20, 40] According to Csapo, Corner, and their co-workers,[49-51] progesterone does not maintain a quiescent uterus, as is widely believed, nor does it reduce the potential working capacity of the uterus. This group believes, on the basis of considerable evidence, that progesterone blocks both excitation and propagation of coordinated activity, and that this is its chief function in maintaining pregnancy.

In the present experiments, progesterone did not maintain pregnancy efficiently in spayed rats, because many fetuses were damaged *in utero*. Addition of a daily low dosage of estrogen not only reduced the necessary progesterone maintenance dose by a factor of 5 to 10, but prevented pressure damage to fetuses. This estrogen effect might be accomplished: (1) by stimulating endogenous progestagen production and release by other endocrine organs, for example, adrenal and placenta; (2) by reducing uterine tonus; or (3) by maintaining a proper ratio of uterine tissue to fetal growth.

The first hypothesis appears unlikely, as the progesterone dosage reduction allowed by concomitant estrogen is of such great magnitude that one would expect estrogen alone to maintain pregnancy if it were capable of stimulating production of such significant quantities of a progestational substance. The latter two hypotheses appear more attractive, since Csapo and Corner[50] have reported a higher uterine tonus in progesterone-dominated than in estrogen-dominated uteri, and it is generally agreed that estrogen is the most potent stimulator of uterine growth.

In spayed mice, estrogen injection did not appreciably reduce the dose of progesterone necessary to maintain pregnancy,[12] nor was exogenous estrogen necessary for duplication of structural and functional changes necessary for parturition.[8, 19, 20] However, Hall[12] reported that relaxin administration reduced the dosage of progesterone necessary for pregnancy maintenance in spayed mice. Since relaxin effects are very slight in ovariectomized, nonestrogenized nonpregnant mice,[52] it is reasonable to assume that there may be a significant endogenous supply of estrogen in the spayed pregnant animal. Thus, the data suggest, but do not prove, that there may be an adequate extraovarian source of estrogen in pregnant mice but not in pregnant rats.

The relationship of cervical dilatability to pregnancy maintenance and the factors actually responsible for its progressive softening in the latter stages of pregnancy remain unclear. However, results in spayed rats suggest that estrogen-progesterone therapy adequate for pregnancy maintenance prevents or reduces the expected increase in dilatability, whereas addition of relaxin to the treatment induces marked cervical softening. It is perhaps significant that extreme dilatability induced by high dosages of relaxin did not precipitate abortion, and this fact suggests that the "incompetent cervix" of human pregnancy[53, 54] may be related to upright posture and is not a factor in the creeping rodents.

Factors influencing parturition. Csapo[49] reports evidence obtained in rabbits that is interpreted to indicate that a drop in progestin levels is necessary to restore uterine function for parturition. Progestin withdrawal is thought to remove the "block" against contractile stimulants such as oxytocics and the block against propagation of coordinated contractile waves. Steinetz *et al.*[8] confirmed the progesterone block in the mouse, but reported that this block against oxytocics and coordinated contractions could be removed by injecting relaxin rather than withdrawing progesterone. They also observed that a highly receptive delivery mechanism (to exogenous oxytocin stimulation) could exist without the occurrence of parturition, and they suggested that other factors as yet unknown must be involved in the initiation of delivery. Further evidence that this may be so derives from the observation that continued daily dosages of 1 mg. progesterone uniformly prolonged pregnancy in ovariectomized mice, but generally did not have this effect in intact mice. The literature includes relevant observations that removal of the ovaries at term[55] or hypophysectomy in the last trimester[56] has prolonged pregnancy in rats. Csapo[49] reported that progestin domination was greatest in the myometrium adjacent to placenta, and he interpreted this as evidence of placental secretion of progestin. An alternative possibility is that the placenta may concentrate circulating progestin, which could explain the high incidence of retained placentae found in progesterone-maintained spayed rats following delivery of fetuses when therapy was withdrawn.

Although softening and dilatation of the uterine cervix are obviously necessary to parturition, it is impossible to ascertain from the data whether this is an active or passive process. Uyldert and DeVaal[57] long ago observed that surgical separation of the cervix from the uterus in intact pregnant rats did not prevent the normal increase in dilatability with advancing pregnancy.

Thus, pressure from the uterine contents was eliminated as a factor, and the results were suggestive of an endocrine effect. However, in the experiments reported here, increased cervical dilatability was observed in spayed pregnant rats (1) when insufficient estrogen or progesterone was administered to maintain a full complement of fetuses, (2) when estrogen and progesterone were withdrawn, or (3) when relaxin was injected.

The "spontaneous parturition" observed in spayed rodents by Smithberg and Runner,[19, 20] Hall,[12] and in this report, induced most successfully by progesterone withdrawal following relaxin treatment, resembled what might be termed abortion at term. Survival of young was generally poor; litter destruction and failure to nurse were commonly observed. Relaxin pretreatment appears to insure more effective and complete expulsion of uterine contents than simple progesterone withdrawal, but the smooth sequence of events leading to the large healthy litter of the normal animal has not yet been duplicated by injected hormonal substitutes for the ovary.

SUMMARY

Three methods for the bioassay of relaxin were briefly described and evaluated: (1) guinea pig pubic symphysis palpation; (2) mouse interpubic ligament direct measurement; and (3) *in vitro* inhibition of mouse uterine segment motility.

Using various combinations of relaxin, estrogen, and progestagens in spayed rats and mice, it was observed that uterine weight (wet and dry), uterine glycogen, and uterine water content could be varied independently of each other.

Estrogen caused only a transitory rise in uterine water content, whereas relaxin or ethylnortestosterone maintained high uterine water levels with continued therapy. Increased estrogen treatment reduced the effects of relaxin and ethylnortestosterone on uterine water content.

Progesterone was antiuterotrophic in estrogen-primed spayed rats and mice, whereas relaxin and ethylnortestosterone were each uterotrophic. However, augmentation occurred when relaxin and progesterone were injected concomitantly.

Relaxin treatment induced a significant increase in cervical dilatability in spayed estrogen-primed rats. Increased estrogen decreased cervical dilatability and reduced the effect of relaxin. Combinations of relaxin and progesterone or relaxin and ethylnortestosterone gave the greatest increase in dilatability. Relaxin or progesterone induced an increase in cervical dilatability in estrogen-primed *mice*.

A dosage of 10 mg. progesterone per day was necessary for complete pregnancy maintenance in rats spayed on the fifteenth day. This dosage could be reduced to 1 to 2 mg. daily by concomitant injection of 1 μg. estradiol 17β.

An inverse relationship between pregnancy maintenance and cervical dilatability was observed. The cervix did not soften in spayed pregnant rats on adequate progesterone or estrogen-progesterone therapy unless relaxin was also injected.

Progesterone "blocked" the delivery response to oxytocin in intact and ovariectomized mice. Relaxin restored the responsiveness to oxytocin of the delivery mechanism in spayed mice treated with progesterone.

A lower daily dosage of progesterone was required to prolong pregnancy in ovariectomized than in intact mice.

Relaxin did not prevent progesterone-prolonged pregnancy in mice, but maintained oxytocin responsiveness when tested on the twenty-third day. Fetal survival was also improved by the combined therapy.

"Spontaneous parturition" at term was most successfully accomplished in spayed rats and mice by relaxin pretreatment and progesterone withdrawal near term. Progesterone withdrawal alone led to retained placentae in rats, and to high *in utero* fetal mortality, maternal toxicity, and prolonged pregnancy in mice. Survival of litters was very poor regardless of treatment.

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THE ACTION OF RELAXIN ON THE UTERUS OF THE RAT, MOUSE, AND RABBIT*

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Recent investigations have revealed a new biological property for relaxin. This property is concerned with the ability of relaxin to influence the morphology, physiology, and biochemistry of the uterus. Within the past few years it has been demonstrated that relaxin depresses uterine contractions in the rat,[1, 2] increases the water content of the uterus of the sow[3] and rat,[4] increases the glycogen content and dry weight of the uterus of the estrogen-primed rat,[5] acts synergistically with estradiol in increasing uterine dry weight in the rat,[6] and inhibits decidual growth in the rat.[7]

The studies described in this paper were undertaken in an attempt to expand our knowledge of this phase of the physiology of relaxin. This investigation is concerned with the interrelationship of estradiol, relaxin, and progesterone and with the influence of relaxin on the (1) estrogen-induced uterine water uptake, (2) the progesterone-maintained deciduoma in the rat and mouse, and (3) the progestational reaction in the mature and immature rabbit.

Material and Methods

The uterine water studies were carried out on immature rats of the Sprague-Dawley strain. All animals were deprived of food, but were placed on a 10 per cent glucose solution ad libitum at 21 days of age and treated with the hormone at 24 days of age. Relaxin‡ was injected subcutaneously in a water solution, and the animals were sacrificed 6 hours later. The uteri were removed, weighed on a torsion balance, dried *in vacuo* for 24 hours at 60° C., and then reweighed in order to obtain the dry weight and the percentage of water.

Adult mice and rats of the Rockland Swiss and Sprague-Dawley strains, respectively, were used in the decidual reaction. The rats were castrated and, 1 week later, treated with 1.0 μg. estrone daily, in oil, for 4 days. This was followed by 1.0 mg. progesterone in oil daily for 9 days. One horn of the uterus was exposed on the fifth day of treatment with progesterone, and 1 mg. histamine dihydrochloride in aqueous solution was injected into the lumen of the left uterine horn.[8] Treatment with progesterone was continued and, in addition, treatment with relaxin was started at this time.[8] The rats were sacrificed 24 hours after the last treatment, that is, 24 hours after 9 daily injections of progesterone or 5 daily injections of relaxin. The uterus was then exposed and the 2 horns were weighed separately. Results are expressed as

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‡ The relaxin used in these studies was supplied in the form of Releasin by Robert L. Kroc, Warner-Lambert Research Institute, Morris Plains, N. J.

the percentage of increase in weight of the treated horn: that is, the weight of the histamine-injected horn minus the weight of the control horn divided by the weight of the control horn and multiplied by 100. The mice were treated in an identical manner except that the animals were given 0.2 μg. estrone, 0.4 mg. histamine dihydrochloride, and 0.8 mg. progesterone in a suspension.

A modified Corner-Allen and Clauberg test was utilized in the investigation of the effect of relaxin on the progestational reaction in the rabbit. Both castrated adult and intact immature Grey Chinchilla rabbits were used. The animals were pretreated with 5 μg. estradiol daily for 6 days, followed by 5 daily injections of 0.5 μg. estradiol and different dosages of progesterone and relaxin. The animals were sacrificed 24 hours after the last injection, and pieces of uteri were fixed in Bouin's solution. The uteri were examined histologically for the presence of a progestational reaction, which was evaluated for the degree reaction on a − to 4+ scale. In addition, the G/M ratio (glandularis area divided by the total mucosal area) also was obtained.[9]

RESULTS

Uterine Water

Previous results from this laboratory had revealed that relaxin was capable of increasing the water content of the uterine tissue and that this action did not require pretreatment with estrogen. It is of interest to note that the peak response with relaxin occurred in six hours, as is seen with estradiol, but no secondary stimulation of uterine growth was observed.

The present experiment was designed to determine whether any possible interaction with estradiol occurred in this response. Immature rats were treated with estradiol and relaxin, both singly and in combination. The data

TABLE 1

EFFECT OF SIMULTANEOUS ADMINISTRATION OF RELAXIN AND ESTRADIOL ON THE UTERINE WATER CONTENT OF THE IMMATURE RAT

No. of animals	Estradiol (μg.)	Treatment (percentage water)	Relaxin (g.p.u.)	Treatment (percentage water)	Uterine water[*] (percentage)	Significant[†] additive effect
10	0.01	(81.5)[‡]	0.2	(81.0)[‡]	82.5	+
10	0.01	(81.5)	0.5	(80.9)	81.5	−
10	0.01	(81.5)	1	(81.6)	85.1	+
10	0.01	(81.5)	2	(82.9)	85.2	+
10	0.02	(82.4)	0.1	(80.8)	82.4	−
10	0.02	(82.4)	0.2	(81.0)	82.7	−
10	0.02	(82.4)	0.5	(80.9)	82.2	−
10	0.02	(82.4)	1	(81.6)	83.3	±
10	0.02	(82.4)	2	(82.9)	84.9	+

[*] Following treatment with both hormones.

[†] At the 1 per cent level.

[‡] The mean uterine water response to hormones, when administered individually, is indicated in parentheses.

In TABLE 1 reveal an additive effect by estradiol and relaxin on the water content of the uterine tissue. The average per cent of uterine water obtained with 0.01 μg. estradiol was 81.5; the percentages obtained with 1 or 2 g.p.u. of relaxin were 80.9 and 81.6, respectively. Simultaneous treatment with 0.01 μg. estradiol and 1 or 2 g.p.u. of relaxin gave values of 85.1 and 85.2 per cent uterine water, respectively (TABLE 1). These results are significant at the 1 per cent level. Similarly, an additive effect also was obtained with 0.02 μg. estradiol and 2 g.p.u. of relaxin. One g.p.u. of relaxin and 0.02 μg. estradiol also gave an additive effect, but it was not statistically significant.

Progesterone failed to show any additive or augmentative effect with relaxin on the uterine water effect. Actually, the opposite was true, in that 1 mg. progesterone reduced the percentage of water in the uterus from a value of 84.2 for 10 g.p.u. relaxin alone to 82.5 for 10 g.p.u. relaxin and 1 mg. progesterone. In this respect, progesterone inhibited the uterine water imbibition to the same degree as that seen with estradiol.

Decidual Reaction

The initial experiment on the effect of relaxin in the rat decidual test revealed contradictory results (TABLE 2). While 1 mg. progesterone caused a 287 per cent increase in the treated horn, the same dose of progesterone plus 300 g.p.u. relaxin caused a 186 per cent increase in the treated horn. The decrease in the decidual response to progesterone and relaxin as compared to the response obtained with progesterone alone was significant at the 1 per cent level. A dose of 150 g.p.u. relaxin plus 1 mg progesterone gave a response of 291 per cent, which is comparable to that obtained with relaxin alone. Sixty g.p.u. of relaxin, however, caused an increase of 191 per cent, which is significantly less than the increase obtained with progesterone alone. A combination of 0.5 mg. progesterone and 150 g.p.u. relaxin gave a response of 181 per cent, which is not significantly different from the 164 per cent increase obtained with progesterone alone.

In a second experiment, 3 groups of rats were used. The first group received only 1 mg. progesterone, the second group received 1 mg. progesterone

TABLE 2

EFFECT OF RELAXIN ON THE DECIDUAL REACTION IN THE RAT

No. of rats	Body weight (gm.)	Progesterone (mg.)	Uterus			Per cent increase in treated horn	Significant difference from progesterone control
			Relax in (g.p.u.)	Control horn (mg.)	Treated horn (mg.)		
5	223	0.2	—	78.7	131.1	68	
9	214	0.5	—	68.4	175.7	164	
16	228	1.0	—	94.3	379.7	287	
5	218	2.0	—	95.1	476.8	411	
5	215	1	300	218.7	617.9	186	+
6	253	1	150	89.3	351.2	291	—
10	217	1	60	77.8	226.7	191	+
5	216	0.5	150	100.6	288.3	181	—

TABLE 3
RATS—DECIDUOMA

No. of rats	Body weight (gm.)	Treatment (progesterone)	Relaxin (g.p.u.)	Uterine weight		Increase (percentage)
				Control H.	Treated H.	
8	207	1 mg.	—	52.7	157.1	204.0
10	194	1 mg.	300*	90.8	172.9	90.4
10	216	1 mg.	300†	78.4	262.1	234.4

* Crude relaxin; potency 30 g.p.u./mg.
† Purified relaxin; potency 150 g.p.u./mg.

TABLE 4
EFFECT OF RELAXIN ON THE DECIDUAL REACTION IN THE MOUSE

No. of mice	Body weight (gm.)	Treatment (progesterone)	Relaxin* (g.p.u.)	Uterine weight		Increase (percentage)
				Control H.	Treated H.	
14	25.8	0.8 mg.	—	32.8	133.1	305.7
10	29.7	0.8 mg.	50†	37.1	125.0	267.4
5	25.8	0.8 mg.	100†	34.2	162.0	321.4
7	25.1	0.8 mg.	100‡	34.1	83.8	146.3

* Relaxin potency.
† Thirty g.p.u./mg.
‡ One hundred and fifty g.p.u./mg.

TABLE 5
COMPARISON OF TOXIC ACTION FOR CRUDE PREPARATION OF RELAXIN, FORMALDEHYDE, AND BENZOPURPURINE

Treatment	No. of rats	Body weight (gm.)			Adrenal gland (mg. %)		Thymus		Dead during treatment (percentages)
		Start	End	Gain	Mg.	Body weight	Mg.	Mg. %	
Saline	10	71.1	90.1	19.0	26.1	29.0	238.4	264.5	0
HCHO (1.5 %)	10	66.8	83.4	16.6	26.8	32.1	163.9	196.5	0
Benzopurpurine (1 %)	10	70.5	88.5	18.0	25.2	28.5	239.1	270.1	0
Relaxin (crude, 30 u./mg., and 10 mg.)	8	66.3	74.9	8.6	28.8	38.5	78.4	104.6	20

plus 300 g.p.u. of a crude relaxin, and the third group received also 300 g.p.u., but of a highly purified relaxin (TABLE 3). Progesterone at 1 mg. caused a 204 per cent increase in the treated horn, whereas the crude relaxin and progesterone caused a 90 per cent increase, and in the third group (treated with progesterone and the purified relaxin) there was a 234 per cent increase. Thus the crude relaxin produced a marked inhibition, whereas the purified material had no significant effect.

Contradictory results also were obtained in the mouse decidual reaction. In 2 instances, dosages of 50 and 100 g.p.u. relaxin failed to influence significantly the action of progesterone on the decidual growth (TABLE 4). However, in 1 instance, a 52 per cent inhibition of the progesterone-stimulated deciduoma was obtained with 100 g.p.u. of relaxin.

These results led us to suspect the possible involvement of the adrenal corticoids, and an experiment was devised to determine whether the adrenal cortex might be stimulated under the conditions of this experiment and hence interfere with the reaction. Four groups of immature male rats weighing from 60 to 75 gm. were injected daily with 0.2 ml. saline, 1.5 per cent formaldehyde, 1 per cent benzopurpurine* and crude relaxin (10 mg./2 ml.) for 5 days and were sacrificed 24 hours later (TABLE 5). The benzopurpurine was included, since it has been used as a depot agent for relaxin. Two of the 10 rats treated with crude relaxin died during the course of treatment. In both instances, the site of injection showed areas of edema, necrosis, and nodule formation. This condition was also apparent in all the rats of this group at post-mortem. The relaxin-treated rats showed the smallest weight gain (8.6 gm., as opposed to 19.0 for the saline controls), adrenal hypertrophy (38.5 mg. per cent body weight, as compared with 29.0 mg. per cent for the controls), and marked thymus atrophy (104 mg. per cent, as compared with 264.5 mg. per cent for the controls). Interestingly enough, the relaxin-treated rats gained less weight than the formaldehyde-treated group and showed a more severe thymus atrophy. The benzopurpurine-treated rats showed no marked changes from the saline-treated controls.

Progestational Reaction

Adult castrated rabbits were pretreated with 5 μg. estradiol daily for 6 days, followed by 0.5 μg. estradiol daily plus progesterone, and with and without relaxin for 5 days. Treatment with progesterone alone caused a marked progestational reaction at the daily dose of 0.2 mg. (3+ reaction) and gave negative results with 0.1 mg. progesterone (TABLE 6). The addition of 300 g.p.u. relaxin to the treatment with 0.1 mg. progesterone gave a 2+ progestational reaction. An increase in the dosage of relaxin led to a marked increase in the progestational reaction, which was verified by the G/M ratio. A ratio of 0.346 was obtained with 0.1 mg. progesterone, and a ratio of 0.464 was observed with 0.1 mg. progesterone and 300 g.p.u. relaxin. Increase in the dose of relaxin to 900 g.p.u. further increased the ratio to 0.534. No further increase in the ratio was noted with 1200 and 1500 g.p.u. relaxin.

A positive progestational reaction of 3+ was obtained with 0.2 mg. progesterone alone with a G/M ratio of 0.492. Treatment with 0.2 mg. progesterone and 600 to 3000 g.p.u. relaxin failed to show any marked histological change, but the G/M ratio was increased to 0.644, as compared to 0.492 for 0.2 mg. progesterone alone.

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* Also supplied by Robert L. Kroc.

TABLE 6

POTENTIATION OF THE ACTION OF PROGESTERONE BY RELAXIN ON THE PROGESTATIONAL REACTION IN THE MATURE RABBIT

No. of rabbits	Progesterone (mg./day)	Relaxin (g.p.u./day)	Progestational reaction	G/M ratio
1	—	1000	−	—
2	0.05	—	−	0.245
5	0.1	—	− − − − ±	0.346
1	0.2	—	3+	0.492
2	0.1	300	2+; 2+	0.464
2	0.1	600	−; 2+	—
3	0.1	900	1+; 3+; 3+	0.534
1	0.1	1200	2+	0.456
1	0.1	1500	3+	0.448
1	0.2	600	3+	—
2	0.2	1500	2+; 4+	0.644
1	0.2	3000	3+	—

TABLE 7

POTENTIATION OF PROGESTERONE BY RELAXIN IN THE PROGESTATIONAL REACTION IN THE IMMATURE RABBIT

No. of rabbits	Progresterone (mg./day)	Relaxin (g.p.u./day)	Progestational reaction	G/M ratio
1	—	—	−	0.216
1	0.02	—	−	0.208
1	0.02	600	−	0.214
1	0.02	1200	−	0.253
1	0.02	2400	−	0.331
5	0.05	—	−; −; −; ±; −	0.233 0.295 0.241
2	0.05	600, 600	−; +	0.230 0.380
2	0.05	1200	2+	0.383
2	0.1	—	− − ±	0.300 0.308
1	0.1	150	± to +	0.363
1	0.1	300	+	0.361
1	0.2	—	4+	0.513

The data from the immature rabbits confirm the results obtained above (TABLE 7). A daily dose of 0.02 mg. progesterone failed to produce an unequivocal progestational reaction, whether it was given alone or in conjunction with relaxin (600 to 2400 g.p.u. per day). However, significant changes in the G/M ratio were caused by the addition of relaxin to the progesterone treatment. The G/M ratio was 0.216 for the control animal, and 0.216 for the rabbit treated with 0.02 mg. progesterone. No significant change appeared following additional treatment with 600 g.p.u. relaxin (G/M ratio of

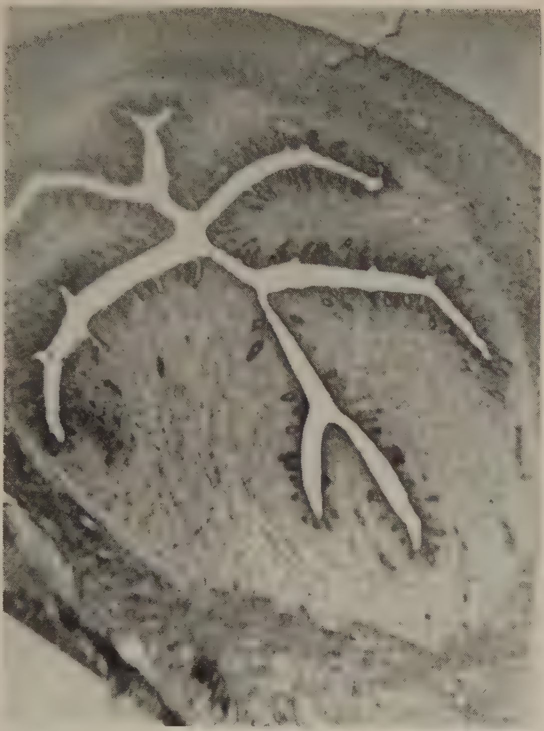

FIGURE 1. Cross section of the uterus of an adult rabbit treated with 0.1 mg. progesterone. Note the lack of any marked progestational response.

0.214), but the addition of 1200 g.p.u. relaxin gave a G/M ratio of 0.253, and 2400 g.p.u. a G/M ratio of 0.331.

Treatment with 0.05 mg. progesterone gave a negative progestational response in 5 rabbits and a G/M ratio of 0.233 to 0.295. Additional treatment with 600 g.p.u. relaxin gave 1 negative and 1 positive reaction and a G/M ratio of 0.230 and 0.380, respectively, while 1200 g.p.u. gave a 2+ reaction and a G/M ratio of 0.383. An increase in the progesterone dosage to 0.1 mg. was negative in 3 animals, with an average G/M ratio of 0.304. The addition of 150 g.p.u. relaxin gave a questionable positive response and a G/M ratio of 0.363, but 300 g.p.u. gave a positive response and a G/M ratio of 0.361.

The degree of potentiation exhibited by relaxin may be seen in FIGURES 1 and 2. FIGURE 1 is a cross section of the uterus of an adult rabbit treated with 0.1 mg. progesterone; FIGURE 2 is a section of the uterus of a rabbit treated with 0.1 mg. progesterone and 600 g.p.u. relaxin. It is apparent that this dose of progesterone produced little to no progestational proliferation, whereas the addition of 600 g.p.u. resulted in a 2+ reaction.

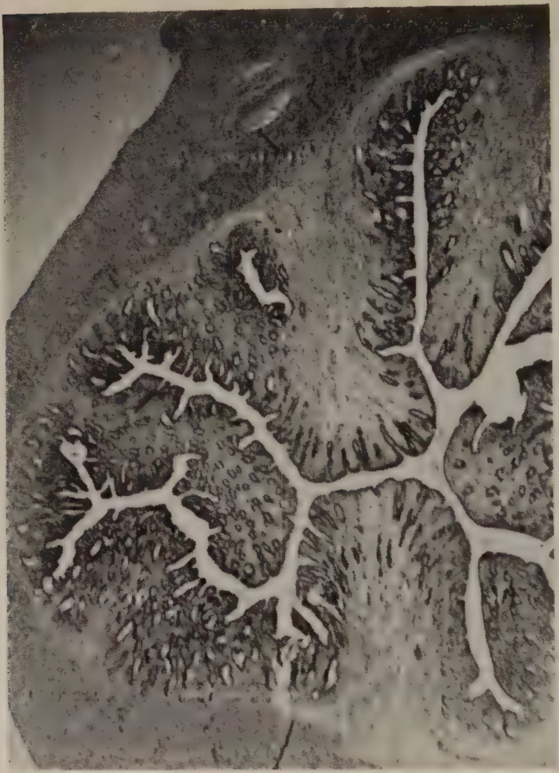

FIGURE 2. Cross section of the uterus of an adult rabbit treated with 0.1 mg. progesterone and 600 g.p.u. relaxin. Note the marked progestational response.

DISCUSSION

The results of these experiments on the progestational reaction in the rabbit indicate without question that relaxin possesses the ability to potentiate the action of progesterone in the progestational reaction. Relaxin itself had no effect, whereas a combination of relaxin and progesterone produced a progestational reaction at a dose level of progesterone that was inadequate to induce a positive response by itself. Even with an effective dose of progesterone, the extent of the reaction was increased by additional treatment with relaxin.

The present data are inadequate for a quantitative analysis on the degree of potentiation, although the results from the G/M ratio and the minimum dose needed for a progestational reaction after treatment with progesterone as compared with progesterone and relaxin tend to suggest a potentiation of fivefold or more. However, more data are needed here before a definite statement can be made. In addition, the data suggest also that a definite quantitative relationship exists between the amounts of relaxin and progesterone and the degree of potentiation obtained.

The present data from the experiment on the decidual reaction in rats and mice fail to show any clear-cut and definite potentiation of progesterone by relaxin. It should be pointed out, however, that these experiments are still in a preliminary state. Thus far the results indicate that relaxin may not inhibit per se the action of progesterone. The data reveal that relaxin either has no effect or may either inhibit or potentiate progesterone. In searching for a possible answer to these extremely variable results, we explored the possibility that the variable results were due to an interfering factor. The possible interference by the adrenal cortex was considered, since it has been shown that the glucocorticoids inhibit the decidual reaction.[10] Experiments with crude relaxin revealed that impure preparations could readily activate the pituitary-adrenal axis and thus result in an inhibition.

Frieden and Velardo[7] report that the potentiation factor in relaxin is a contaminant and that the inhibitory factor noted in the decidual response is a specific part of the relaxin hormone. Our results do not contradict this, except to indicate that inhibition has not been seen in the rabbit, nor has it been demonstrated clearly in the rat or the mouse. Actually, the results obtained with the rabbit species indicate a potentiation of progesterone, while the occasional inhibition seen in the rat and the mouse could be a nonspecific effect involving the pituitary-adrenocortical axis.

It is of interest to note also the similarity in action of estradiol and relaxin on the water content of the uterus. Both hormones produced their maximum effect on uterine water in six hours, and the action of each hormone was additive within certain limits. Furthermore, progesterone produced a partial inhibition of the water response obtained after either estradiol or relaxin.

SUMMARY

The present results indicate that relaxin preparations possess the ability to potentiate the action of progesterone on the progestational reaction in the rabbit. An approximately fivefold increase in the effectiveness of progesterone was obtained by the addition of relaxin. Results on the decidual test in rats and mice were extremely variable, indicating both inhibition and no effect at all. A possible explanation is presented for these variable results. Relaxin was shown to act in an additive manner with estradiol on the water content of the uterine tissue.

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SOME CLINICAL OBSERVATIONS OF RELAXIN IN OBSTETRICS

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Relaxin, a nonsteroidal ovarian hormone, has been the subject of considerable attention in the recent past. There have been indications that relaxin is capable of inhibiting premature labor,[1] enhancing and accelerating normal labor,[2] and causing the unripe cervix of term pregnancy to become soft and responsive to the forces of labor (M. Stone, personal communication). Some of these observations would appear to be contradictory. Some of the series reported lack adequate controls, so that proper evaluation is difficult. Four studies have been undertaken to attempt to clarify the effects of relaxin* during various conditions in the pregnant woman. An attempt has been made to utilize adequate controls in each of these studies.

Effect of Relaxin on Normal Labor

A double blind study was conducted on 216 patients in normal labor. Upon admission to the hospital, alternate patients (109) were given relaxin in two forms, 20 mg. in saline solution and 20 mg. in a gelatin suspension (repository); the remaining patients (107) were given a placebo similarly packaged. The identity of neither form was known to any of the investigators until the study had been completed. Patients received the medication in various stages of the first stage of labor (TABLE 1). A review of the duration of labor before and after medication, as well as the total duration of labor, indicates that there was almost no difference in the various categories, whether the patients received relaxin or the placebo.

Folsome *et al.*[1] have indicated that patients receiving relaxin sometimes have a less painful labor. Although it is difficult numerically to evaluate the different medications and dosages employed for analgesia and sedation during labor, there would appear to be no difference in the amounts required by the two groups in this series.

Effect of Relaxin on the Unripe Cervix

Zarrow and Money,[3] Graham and Dracy,[4] and others have shown that, in certain animals, cervical changes that resemble those seen at parturition can be produced with relaxin. M. Stone (personal communication) and Birnberg and Abitbol[2] have indicated that induction and stimulation of labor can be accomplished more readily by the use of this substance. They claim that this is the result of softening of the cervix.

An attempt has been made to determine accurately the effect of relaxin on

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* The preparation used in this study was Releasin, supplied by Warner-Chilcott Research Laboratories, Morris Plains, N. J.

TABLE 1

Cervix dilatation (cm.)	Relaxin			Control		
	Hours labor before	Hours labor after	Total number of patients	Hours labor before	Hours labor after	Total number of patients
Primiparas						
0–2	7.7	11.5	19.19 (24)	8.1	13.0	21.1 (20)
3–4	9.6	7.7	17.25 (16)	8.9	8.2	17.1 (15)
5–6*	10.1*	8.3	18.37 (8)	7.2	7.4	14.6 (5)
Total (average)	8.8	9.6	18.4 (48)	8.5	10.4	18.9 (40)
Multiparas						
0–2	9.7	7.0	16.7 (19)	5.9	5.5	11.4 (31)
3–4	7.5	3.5	11.0 (31)	8.7	4.8	13.5 (28)
5–6	6.5	4.1	10.6 (11)	3.8	1.6	5.4 (8)
Total (average)	8.0	4.7	12.7 (61)	6.8	4.7	11.5 (67)

* Two patients had 7- to 8-cm. dilatation.

TABLE 2

Patients treated with relaxin (21)	
No change in cervix	13
Slight change in cervix	6
Marked change in cervix	2
Average duration of labor	18.7 hr. (longest, 31 hr.; shortest, 6 hr.)
Type of delivery:	
Spontaneous or low forceps	20
Cesarean section (cephalopelvic disproportion)	1
Untreated patients (17)	
No change in cervix	9
Slight change in cervix	7
Marked change in cervix	1
Average duration of labor	17.9 hr. (longest, 27 hr.; shortest, 4 hr.)
Type of delivery:	
Spontaneous or low forceps	16
Mid forceps	1

the cervix of the pregnant woman at term. Three hundred and twelve primiparas were examined at weekly intervals during the last 6 weeks of pregnancy. From these 38 were selected who, when 7 to 10 days from term, showed a cervix that was uneffaced, firm, and closed. These cervices were judged to be "unripe." Twenty-seven of these patients were admitted to the hospital and given relaxin in doses of 20 to 40 mg. 2 to 4 times a day. They were examined every other day in an effort to determine what cervical changes, if any, occurred. A system of grading cervical softening, effacement, and dilatation of the internal os was devised. The remaining seventeen patients received no medication and were used as controls. TABLE 2 shows the results of this study; there was no consistent or appreciable change in the cervices of either of the two groups, or in the quality or character of eventual labor and delivery.

Effect on Premature Labor

Perhaps the clinical application of relaxin in premature labor has received the most attention. Abramson and Reid[5] first reported on 5 cases of pre-

TABLE 3

Premature labor relaxin		Period of gestation (weeks)	
Multiparas	23	32–36	24
Primiparas	13	28–31	10
		Less than 28	2
Total cases	36		

Probable cause of premature labor	
Premature rupture of membranes	8
Premature separation of placenta	6
Toxemia	2
Intrauterine fetal death	3
Unknown	17
Lobar pneumonia	1

Degree of dilatation at time of relaxin administration	
0–2 cm.	19
3–4 cm.	9
5–6 cm.	4
6 cm.	1
Unknown	4

Labor ceased	4
Labor inhibited	2
Number of labors uninfluenced by relaxin	31 (84 %)
Number of patients delivered prematurely following relaxin	33 (89 %)

TABLE 4

Premature labor controls		Period of gestation (weeks)	
Multiparas	15	32–36	22
Primiparas	17	28–31	6
Total cases	32	Less than 28	4

Probable cause of premature labor	
Premature rupture of membranes	8
Premature separation of placenta	3
Toxemia	3
Intrauterine fetal death	1
Unknown	16
Polyhydramnios	1
Number of premature labors uninfluenced by treatment	31 (96 %)

mature labor halted by relaxin. This was followed by the work of Folsome and others, who gave confirmatory evidence of these initial findings. In the present study, 37 patients in labor prior to the thirty-sixth week of gestation were given relaxin in doses ranging from 20 to 1000 mg. intramuscularly and by dilute intravenous infusion. Thirty-two patients selected alternately were treated only with sedation and bed rest and were utilized as controls. TABLES 3 and 4 show the results of this study. It will be noted that the majority of those patients given relaxin were in the early stages of labor at the time the drug was given. In only 4 instances did labor cease. Careful evaluation of 2 of these cases showed a lack of the regularity of the contractions expected in true labor. In 2 other instances labor appeared to be inhibited for a short time, but soon resumed and resulted in premature delivery.

In the control group there was one patient whose labor ceased, and this case must be considered an instance of false labor.

From these results one is forced to conclude that relaxin has little influence on the uterine contractions of premature labor.

Conclusions

The studies presented here are essentially of a negative nature. Steinetz *et al.*,[6] as well as many others, have shown that the effects of relaxin in laboratory animals can be altered when combined with progesterone and estrogen. This would lead to an assumption that relaxin (like other hormones) does not act entirely independently, but is a part of a hormone complex that is not yet clearly understood. Until such time as its role is clarified, the clinical applications of this drug cannot be evaluated efficiently. Considerable basic research will be necessary to achieve this end.

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LUTREXIN IN THE TREATMENT OF PREMATURE LABOR

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Since 1903, when Ludwig Frankel of Breslau showed that the removal of the corpus luteum in the pregnant rabbit caused it to abort, there has been a more or less constant search by many investigators to find a substance in the corpus luteum or ovary that could be used in the treatment of habitual and threatened abortion and premature labor. Since the paper by Krohn *et al.*,[1] there have been many papers that have shown that various extracts of the corpus luteum are of value in the treatment of threatened abortion and premature labor. In 1942 Falls *et al.*[2] showed relatively good results in over 600 cases with an injectable corpus luteum extract. In 1950 Krantz *et al.*[3] showed that an aqueous extract of the sow corpus luteum had the ability to diminish uterine tone and abolish contractions of the guinea pig uterus *in situ*. I[4] used the same material to determine its effectiveness in the treatment of dysmenorrhea. Jones and Smith[5] also used the substance in the treatment of dysmenorrhea. Majewski and Jennings[6a, 6b] continued the work with this substance in the treatment of clinically diagnosed cases of premature labor. It is my belief that threatened abortion differs from premature labor only in the length of gestation. Threatened abortion presents a problem in differentiating between a true threatened abortion and an incomplete abortion while, in premature labor, it is difficult to differentiate between premature labor and normal Braxton Hicks contractions. Because many different etiological factors may cause the premature emptying of the uterus, one can expect difficulty in finding one type of treatment to take care of all the factors. All the theories as to the mechanics of the onset of labor are hypothetical. Some feel that the increasing size of the uterus may cause growing irritability and produce spontaneous labor. Other theories are concerned with the environment of the cervix and lower uterine segment of the uterus near term, the carbon dioxide content of the blood, and senility of the placenta. Lash and Lash[7] feel that the incompetent os is a factor in premature labor, and Trythall[8] has achieved 55 per cent good results with the use of Lutrexin in the treatment of the incompetent os. Others feel that psychic or physical trauma may be the trigger mechanism that produces premature labor. Since Snyder[9] has been able to prolong the duration of pregnancy in the rabbit by inducing ovulation near term, and since Heckel and Allen[10] have used progesterone to delay parturition of the rabbit, the hormonal initiation of labor probably is a good factor.

In this series of 54 cases of premature labor between 24 and 34 weeks' gestation treated with Lutrexin, the presence of at least 2 of the following 4 conditions was necessary for classification as premature labor: (1) regular uterine contractions causing definite discomfort to the patient; (2) at least 2-cm. cervical dilatation in a primipara and at least 3-cm. dilatation in a multipara; (3) some vaginal bleeding or a bloody show; and (4) rupture of the bag of water. Uterine contractions with progressive descent of the head but without cervical dilatation or bleeding were considered normal Braxton Hicks contractions and were not included in this series of 54 cases.

The treatment consisted of bed rest plus 3000 units of Lutrexin* orally every hour until the symptoms subsided, and then 3000 units 4 times a day for 2 days. After this the patient was permitted to be up, and received 1000 units 3 times a day until viability was reached (36 weeks). The patient was not awakened at night for therapy. In 36 of the 54 cases the patient was given a barbiturate at night for sleep; no sedation was used in the daytime except in 6 quite disturbed cases that required tranquillizers.

Of the 54 cases, there were 14 failures; of these 14 failures, 2 fetuses survived—one weighed 3 lb. 14 oz., and one weighed 2 lb. 15 oz. In the 12 that did not survive, the weight varied from 1 lb. 12 oz. to 3 lb. 15 oz. Two were stillborn. A case was classified as a failure if the patient continued to have pains and terminated the pregnancy. The 40 that were classified as successes continued with the pregnancy for at least an additional 28 days. In the group of failures, 4 were first seen with 4-cm. dilatation and ruptured membranes, and all terminated their pregnancies in less than 12 hours. In the salvage group of 40 cases, 5 had ruptured membranes, but with very little dilatation of the cervix (less than 2 cm). Thirteen patients of this series had been hospitalized previously as cases of threatened abortion between the tenth and sixteenth weeks of gestation. Four of the 54 cases proved to be cases of placenta praevia that began to bleed between 24 and 27 weeks. Diagnosis of placenta praevia was confirmed at the time of cesarean section between 36 and 38 weeks. One case of placenta praevia threatened to abort at 14 weeks and again at 19 weeks. In this series there were 30 primiparas and 24 multiparas. Of the 54 cases, 32 had lost at least 1 pregnancy and 2 had lost 3.

Summary

In this series of 54 cases, premature labor was stopped in 40 cases (74 per cent) for at least 28 days. I feel that, by delaying delivery for 28 days or more, we have enabled many fetuses to survive that otherwise would have died because of prematurity.

It appears that the substance that inhibits uterine contractions and is of value in the treatment of premature labor, threatened abortion, and dysmenorrhea has not been completely isolated, but is found in Lutrexin in therapeutic amounts.

There were no untoward effects noted from this therapy.

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EFFECTS OF RELAXIN ON THE NONPREGNANT AND PREGNANT UTERUS

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Relaxin, a nonsteroid, water-soluble, ovarian hormone, was first described by Hisaw in 1926.[1] In the subsequent three decades, intensive study of this hormone has resulted in purification and analysis of its active fractions. The background of relaxin research has been described by Hisaw and Zarrow,[2] and its biochemistry by Frieden and Hisaw.[3]

In recent years *in vitro* and *in vivo* animal studies with potent preparations of relaxin have revealed that this hormone has two specific properties: (1) the inhibition of uterine muscle contractions[4-6] and (2) the reduction of tension in the cervix.[7, 8] Several clinical studies have reported that both these properties can be demonstrated in the human. As a result, therapy with extracts of relaxin has been advocated to inhibit the uterine contractions associated with premature labor[9-13] and dysmenorrhea;[14, 15] in addition, it has been asserted that treatment with relaxin softens the cervix at term and in labor, thereby facilitating both labor and delivery.[16, 17] Two recent studies, however, have not confirmed such effects in the human.[18, 19]

We have undertaken an *in vivo* study of these two properties of relaxin in the human with the intention of re-evaluating the effects, if any, of this hormone.* Two objective study techniques have been employed: (1) a qualitative and quantitative assay of uterine motility via utilization of the Karlson[20] tocometric method of tiny carbon pressure transducers located on a thin intrauterine probe (FIGURE 1) and (2) a double-blind coded relaxin and placebo project.

In the preparations supplied, 1 cc. contained 20 mg. of relaxin. The placebos consisted of solutions of gelatin. The intravenous and intramuscular routes of administration were used exclusively in these studies.

Relaxin and the Nonpregnant Uterus

The effect of relaxin on nonpregnant uterine muscle was studied tocometrically in specific phases of the menstrual cycle (either the menstrual, proliferative, ovulational, secretory, or the premenstrual phases) in 60 normal women. The relaxin was given intravenously in doses varying from 0.5 cc. to 16.00 cc. Although definite inhibition of uterine contractions frequently was observed in each of the 5 phases, the responses were not consistent. When motility suppression did occur, its duration was dependent upon the dose of the drug, the phase of the cycle, and the individual patient's "susceptibility." Repeated doses of the hormone failed to subdue renewed uterine contractility. It was determined that, although relaxin does have the property of inhibiting the myometrial activity of the nonpregnant uterus, the effect is inconstant.

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* The relaxin used in this study was provided by the Warner-Lambert Research Institute, Morris Plains, N. J., and the National Drug Company, Philadelphia, Pa.

Relaxin and Dysmenorrhea

It has been suggested that relaxin might suppress the hypercontractile myometrium associated with this symptom. A tocometric study of 8 women with essential dysmenorrhea was obtained during painful menses. Relaxin administered intravenously in 20-mg. doses was effective in reducing the amplitude and duration of uterine contractions in 7 of these 8 patients, with concomitant relief of the individual's distress.

The results in these few cases studied tocometrically stimulated further investigation and, in the hope of securing a physiological "cure" of dysmenorrhea, a more extensive clinical study was initiated. A double-blind alternate case program was then established, employing relaxin and a placebo of identical appearance. Forty young women with severe essential dysmenorrhea were selected for study. They were given 1 of the 2 disguised preparations intramuscularly as soon as possible after the onset of menstrual pain. Neither doctor, nurse, nor patient knew which preparation was being given. The relaxin was administered in 40-mg. doses. An attempt to have each patient serve as her own control was established by random administration of either preparation for at least 4 successive painful menses. Thus each girl had 2 relaxin-treated and 2 placebo-treated menses, in random order.

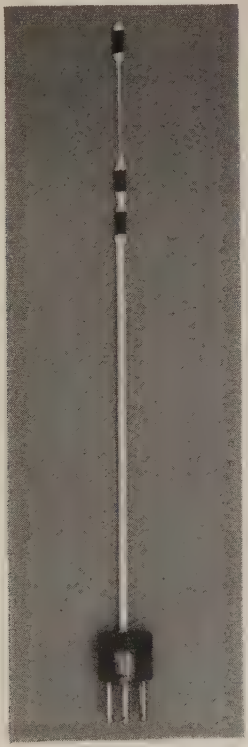

FIGURE 1

Ultimate preparation identification revealed that 32 (80 per cent) of the 40 patients treated were relieved by relaxin in at least 1 painful period; of these 21 (52.5 per cent) were helped in both relaxin-treated menses; and 11 (27.5 per cent) were assisted in only 1 relaxin-treated mensis. The therapeutic implications seemed too good to be true—and they were—for further analysis of these 32 "cured" patients disclosed that 22 of them also were relieved of pain at least once by the placebo. It was sobering to reflect that only 10 patients (25 per cent) were bona fide relaxin "cures." Moreover, 5 of these bona fide cures were relieved in just 1 of the 2 relaxin-treated periods (TABLE 1). Thus, only 1 of every 8 patients treated with relaxin achieved consistent "objective" relief from her dysmenorrhea.

Relaxin and the Pregnant Uterus

Intrauterine tocometry also was employed for appraisal of the action of relaxin on the human uterus in each of the 3 trimesters of pregnancy and in early labor. Relaxin was given intravenously in doses varying from 20 mg. to 320 mg., at an average rate of 2 mg./min. Only 2 of the total of 41 patients studied displayed cessation of myometrial activity following administration of the drug. One patient was 17 weeks pregnant, the other was in early labor at term. It was concluded that, in the doses employed, relaxin had negligible effect on the myometrium of the pregnant uterus.

Relaxin and Premature Labor

Utilization of relaxin has been advocated in premature labor to suppress undesired uterine contractions. Tocometric survey in these patients is desirable, but practical difficulties negate the use of the intrauterine technique. Therefore the double-blind program was instituted; in 50 cases diagnosed as premature labor; alternate patients were treated with 1 of the 2 disguised preparations, relaxin or placebo, both intravenously and intramuscularly. The relaxin dosage per patient averaged 200 mg. intravenously and 160 mg. intramuscularly. In 35 of these women, labor subsided. Eventual code breakdown revealed that 17 of the successes had been treated with relaxin and 18 with the placebo (TABLE 2). There are 2 conclusions: (1) premature labor is difficult to diagnose; and (2) the fact that relaxin was associated with the arrest of contractions does not thereby imply that it was the instrument of such arrest, for placebo therapy was just as effective. As a result of these findings,

TABLE 1

RELAXIN AND DYSMENORRHEA: ANALYSIS OF THE 32 RELAXIN-"CURED" PATIENTS

	Cured in only one mensis	Cured in two menses	Cured at least once
Relaxin	11 (27.5%)	21 (52.5%)	32 (80%)
Placebo	6 (15%)	16 (40%)	22 (55%)
Bona fide relaxin cures	5 (12.5%)	5 (12.5%)	10 (25%)

TABLE 2
RELAXIN AND PREMATURE LABOR

	Labor stopped	Labor continued
Relaxin	17 (68%)	8 (32%)
Placebo	18 (72%)	7 (28%)

TABLE 3
RELAXIN AND THE NONRIPE CERVIX

	Cervix softened	Cervix not softened
Relaxin	5 (20%)	20 (80%)
Placebo	4 (16%)	21 (84%)

one might justifiably assert that relaxin should not be advocated as an effective method of treatment for premature labor.

Relaxin and Promotion of Parturition

The ability of relaxin to facilitate dilatation and expedite effacement of the pregnant uterine cervix has been observed in animals and reported in humans. If true, such an achievement would have broad therapeutic possibilities. A critique of this property of relaxin to soften the cervix was obtained by establishing a double-blind relaxin and placebo study program. Fifty pregnant women at term, with clinically "nonripe" cervices, were treated in the alternate-case fashion with one of the coded preparations. Combined intravenous and intramuscular routes were used. The relaxin doses were the same as those employed for premature labor. A careful pretreatment and posttreatment vaginal examination was performed by one individual to evaluate the condition of the cervix. Forty-one of the 50 patients showed no demonstrable decrease in cervical tension after treatment, despite massive and repeated doses of either preparation. In 9 women, the cervix was adjudged to have undergone definite posttherapy softening. Later preparation-identification revealed that 5 of these 9 cases had received relaxin and that the other 4 had received placebo (TABLE 3). One might conclude that a "ripening" response after relaxin can be ascribed to chance; and that relaxin per se did not demonstrate an ability to soften the cervix at term.

Summary and Conclusions

(1) An objective evaluation of the effects of relaxin on the human uterus *in vivo* has been carried out utilizing tocometric analyses and double-blind placebo studies.

(2) Relaxin was demonstrated to have a definite, albeit inconstant, inhibitory effect on the contractility of nonpregnant myometrium.

(3) Although tocometric study in a few cases revealed that relaxin can subdue the hypercontractile uterine muscle in dysmenorrhea, there was no substanti-

ation of this property in a more extensive double-blind clinical study, indicating that relaxin has no definite role in the therapy of painful menses.

(4) Studies in pregnant women disclosed that relaxin did not hinder the myometrial motility of the gravid uterus.

(5) No change in tension of the pregnant nonripe cervix was observed subsequent to administration of relaxin to pregnant women at term.

(6) Objective investigations of this fascinating hormone supply convincing evidence that, as yet, relaxin has no place in our medical armamentarium for treatment of such conditions as premature labor and dysmenorrhea, or for softening of the nonripe cervix at term.

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RELAXIN IN DYSMENORRHEA AND ITS EFFECT *IN VITRO* UPON MUSCULAR CONTRACTION

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With the advent of the third hormone from ovarian tissue, relaxin, and its availability for clinical use, it became of interest to determine the effect of this hormone upon uterine function in humans. Extensive experimental work has been done with this preparation since it was first extracted and prepared by Hisaw and his group.[1, 2] Experimental work *in vivo* showed that relaxation of uterine contractions could be achieved in animals.[2] Subsequently the material was used in the form of oral tablets and also by parenteral means for the control of dysmenorrhea[3, 4] and premature labor,[5] respectively. We were interested in noting the effect of orally and parenterally administered relaxin in patients with dysmenorrhea. In addition, we were concerned with determining some of its properties *in vitro*, both upon the uterus of rats and the human Fallopian tube.

Dysmenorrhea

The use of relaxin in dysmenorrhea was predicated upon the ability of the material to cause relaxation of smooth muscle, particularly that of the uterus.

Oral studies. The first preparation (ovarian extract L) used was an aqueous nonsteroidal extract prepared from the ovaries of the sow and said to be effective in producing relaxation of the guinea pig uterus. This preparation was similar to that used by other investigators in dysmenorrhea and premature labor.[3-5] The results obtained with this material when administered orally to a series of 20 patients revealed that the compound was no more effective than a placebo. A second preparation, ovarian extract *C*, from the corpora lutea of ovaries obtained from the pregnant sow, also proved to be ineffective when administered orally (TABLE 1). The above negative results were not surprising inasmuch as one would anticipate that any protein extract prepared from biological material would be sufficiently altered by the digestive enzymes to change completely the preparation so that biological activity would be ineffectual after oral ingestion. The fact that these extracts are capable of producing relaxation of uterine musculature of the guinea pig *in vivo* does not mean that similar observation may be anticipated in the human when the same material is administered orally. Reports to the contrary by other workers on the effectiveness of oral ovarian extracts containing uterine-relaxing material in dysmenorrhea[3, 4] are difficult to interpret in view of our data, where the clinical evaluation was accomplished by objective means.

The patients selected for our studies were those with primary, intractable dysmenorrhea. In none of the patients to whom the drug was administered was there any evidence of organic pathology. We know from our own clinical experience that placebo therapy in patients with dysmenorrhea of a mild nature

TABLE 1
COMPARATIVE EFFECTIVENESS OF ORAL OVARIAN EXTRACTS AND PLACEBOS IN PRIMARY DYSMENORRHEA

Preparation	Dose*	Clinical effectiveness		
		Excellent	Moderate	Poor
Ovarian extract *L*	3,000 to 6,000 units q. 3 h.	3	1	16
Ovarian extract *C*	100 mg. q. 4 h.	1	1	11
Placebo	1 tablet q.i.d.	3	1	13

* The medication was administered preceding the onset of pain as nearly as possible or as soon as pain was experienced, and was continued during the entire period of painful menstruation.

may be effective in more than one third of the patients to whom the placebo is administered. The use of any medication in the treatment of dysmenorrhea will be found to be helpful if the choice of patients having the dysmenorrhea is not too selective. This is evident from the long list of preparations that have been advocated for dysmenorrhea in patients with pain of very slight or moderate extent.[6] The patients in this study were those in whom aspirin or the usual analgesic compounds commonly sold over the counter were found to be ineffective. The oral preparations of relaxin were administered in a control double-blind study whereby neither the physician nor the patient was aware of the medication being dispensed. While we have a few placebo reactors, the data in general indicate that these preparations are completely valueless in the treatment of bona fide primary, intractable dysmenorrhea.

The doses used and the data obtained are recorded in TABLE 1. The amount of material employed was considered to be adequate on the basis of data published in the literature. There was no evidence of any undesirable side effects. The material was well tolerated but ineffectual. The patients showing a response to placebo medication at one time failed to respond to placebo medication at another. All the patients in this series have been controlled adequately with respect to their dysmenorrhea by the preovulatory administration of estrogens in doses sufficient to inhibit ovulation. They were treated effectively also with adequate pain relief by the preovulatory administration of methyltestosterone according to the procedures we have described previously.[6, 7]

In view of the failure of oral medication to induce a satisfactory therapeutic effect, a parenteral preparation of relaxin* containing 40 mg. of material per cc. in a delayed absorption menstruum was administered premenstrually to a series of women with primary intractable dysmenorrhea. Two to 3 doses of the preparation were injected intramuscularly at 1- to 3-day intervals 3 to 5 days prior to the onset of menses. This regimen was selected because we felt that attempts to treat the patient on the day pain was initiated would invariably fail. We also thought that accurate timing of the dose would make it completely impossible to administer the medication in women who begin

* The parenteral relaxin used in this study was supplied in the form of Cervilaxin, by William Swain of the National Drug Company, Philadelphia, Pa.

TABLE 2

PARENTERAL RELAXIN IN DYSMENORRHEA

Number of patients	Dose	Clinical response		
		Excellent	Good	Poor
19	1 to 2 cc.*	3	12	4

* 1 cc. = 40 mg.

to menstruate late at night when they are far removed from adequate medical care.

It was believed that, for relaxin to be effective, the preparation should be administered prior to the time that menstrual cramps occurred. In order to obviate this difficulty, the premenstrual administration of the material was initiated before the pain was apparent. We have found this mode of therapy to be a satisfactory method of controlling menstrual pain in 15 of 19 women.

The results in TABLE 2 present our data on the parenteral use of relaxin in dysmenorrhea. An excellent response was recorded in 3 patients in whom no discomfort was observed with the onset of the catamenia. A good response was recorded in 12 patients and was so designated inasmuch as only minimal menstrual pain was experienced. However, the discomfort was not sufficient to interfere with the patient's daily duties. A poor response was noted in 4 patients who obtained no essential relief with parenteral relaxin and sought other analgesic medication or were incapacitated to such an extent that they could not perform their usual activities.

In vitro studies. In order to characterize properly the pharmacological action of relaxin,* preliminary studies on the isolated human Fallopian tube and the isolated rat uterus were done. A more detailed description of our studies will be presented elsewhere. The *in vitro* data indicated that a definite relaxation of contraction of both the human Fallopian tube and rat uterus was achieved.

FIGURE 1 shows the effect of 20 mg. of relaxin when added to the *in vitro* system containing a segment of a human Fallopian tube. A definite relaxation of the normal pattern of contraction was followed subsequently by an increase in tonicity of the musculature. The relaxin was added to the system at Point A. A similar response is recorded in FIGURE 2, where the addition of 40 mg. of relaxin to the *in vitro* system also produced a definite relaxation of contraction of the isolated human Fallopian tube, followed by an increase in muscular tonicity. The relaxin was administered at Point A. Similar effects were observed when relaxin was added to the *in vitro* system containing the isolated rat uterus. The type of response, however, was altered, depending upon the treatment to which the rat had been subjected prior to the removal of the uterine segment.

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* The relaxin material used in the *in vitro* studies was supplied by William Kleinberg of Princeton Laboratories, Princeton, N. J.

All the animals were adult female rats castrated 7 days prior to the administration of the steroid being tested. The effect of different doses of relaxin upon the estrogen-primed rat uterus is depicted in FIGURE 3, where relaxation of uterine contractions was achieved; the degree of relaxation was proportional to the dose of relaxin added to the *in vitro* system when the relaxin dose ranged from 10 to 40 mg. However, one should note that, unlike the tracings obtained with the human Fallopian tube segments, increased muscle tonicity was not observed following the period of muscle relaxation induced by the relaxin. In contrast to the estrogen-primed rats, with the addition of 10 mg. of relaxin to the *in vitro* system containing a uterine segment from a rat primed with progesterone, the anticipated relaxation was achieved. However, this was followed by increased muscle tonicity similar to that noted in the human Fallopian tube studies (FIGURE 4). Point A represents the time of addition of the 10 mg. of relaxin to the system. A similar effect was achieved with a uterus obtained from a rat treated in the same manner, where 20 mg. of relaxin produced relaxation followed by muscle tonicity (FIGURE 5). The relaxin was added at Point A. A more detailed description of these studies will be pre-

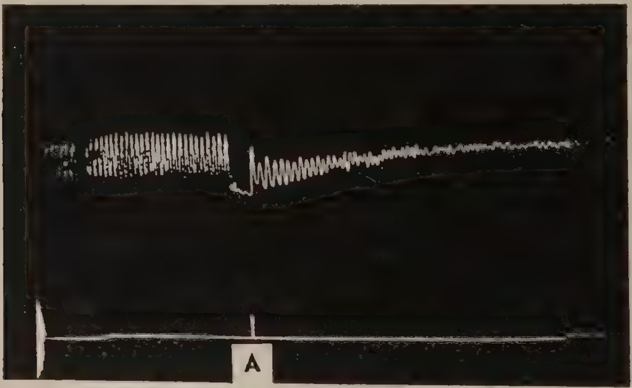

FIGURE 1. Human Fallopian tube contraction pattern before and after the addition of 20 mg. of relaxin (A) to the *in vitro* system.

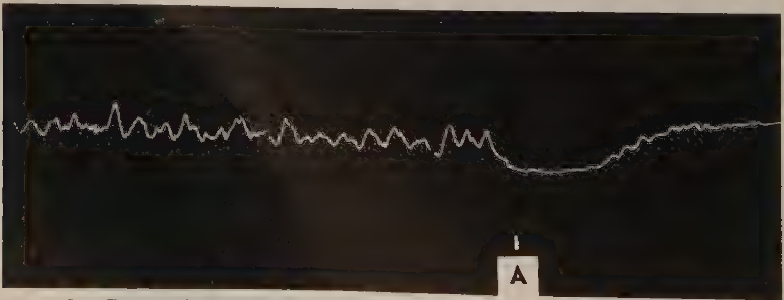

FIGURE 2. Contraction pattern of the human Fallopian tube (postpartum) *in vitro*. A = point of introduction of 40 mg. of relaxin. A = time of drug administration.

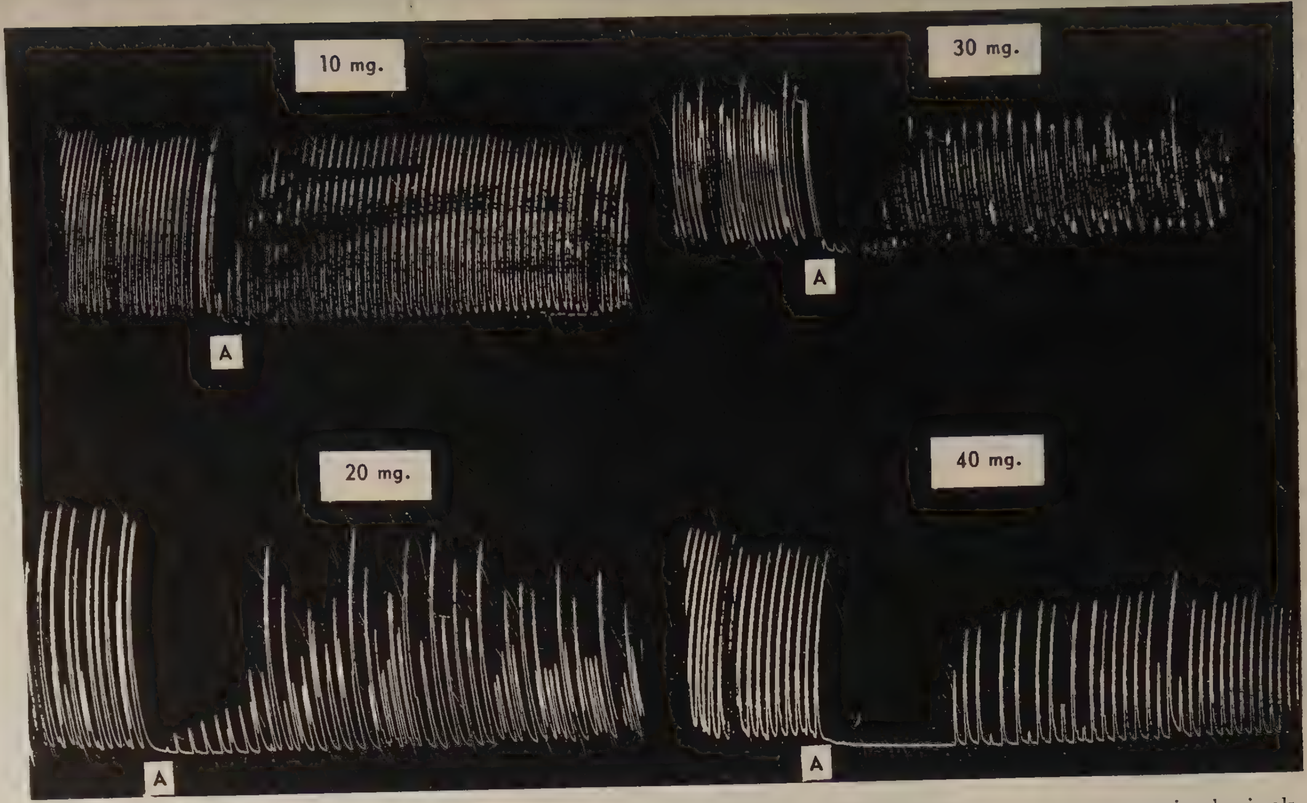

FIGURE 3. Effect of different doses of relaxin upon the *in vitro* contraction of the rat uterus obtained from estrogen-primed animals.

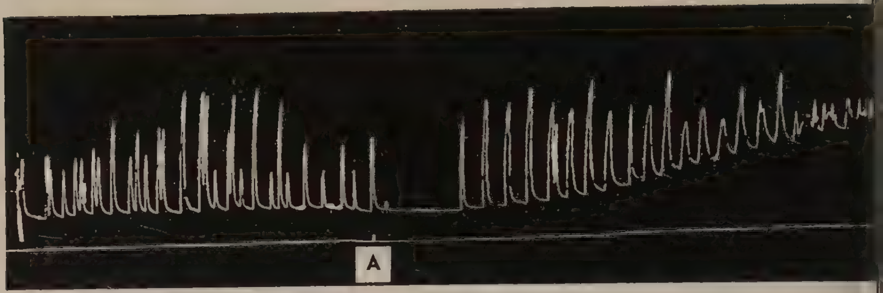

FIGURE 4. Effect of 10 mg. of relaxin upon contraction of the rat uterus obtained from a progesterone-primed castrate. A = 10 mg. of relaxin.

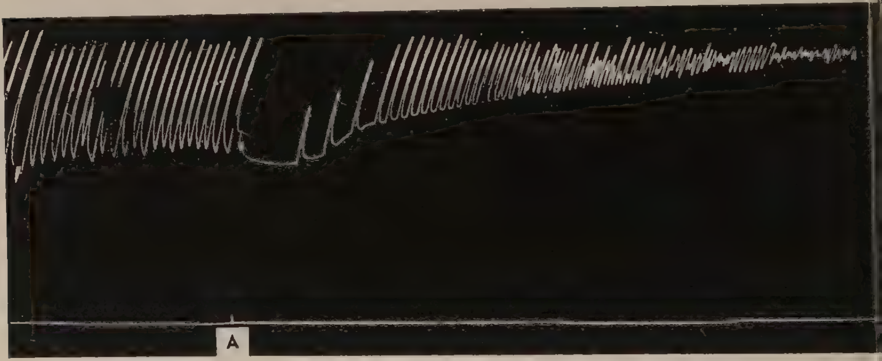

FIGURE 5. Effect of relaxin upon the contraction of the isolated rat uterus obtained from a progesterone-primed castrate female. A = 20 mg. of relaxin added to the *in vitro* system.

sented elsewhere on the effects of relaxin upon the rat uterus obtained from animals primed with steroids of the 19-nortestosterone series, showing effects comparable to those noted after progesterone.

Discussion

The treatment of dysmenorrhea with nonsteroidal preparations could represent an improvement in the management of this distressing clinical entity. The medical management of primary dysmenorrhea has been a very controversial subject. The large number of preparations available and the voluminous literature on the subject attest to the failure of any one procedure to receive unanimous approval. This must be considered as a measure of the general ineffectiveness of the preparation advocated, its recommendation sometimes being based upon the enthusiams of the investigator rather than upon its efficacy or pharmacological action. The more modern approach to the effective treatment of dysmenorrhea has been dependent upon the use of preovulatory administration of steroidal hormones. Estrogens are used primarily to inhibit ovulation.[8, 9] Methyltestosterone, for some unknown reason, produces an dequate therapeutic effect without inhibition of ovulation.[6, 7] The inhibition f ovulation by the use of estrogens, unless infertility is desired, offers an effective lbeit unphysiological mode of therapy, and indicates defeatism on the part of he physician. While the virtues of androgen therapy with respect to efficacy re unqualified, its use has been entered upon with some trepidation by certain linicians because of the feared (but not demonstrable) androgenic effect of the teroid in the doses used. The availability of a nonsteroidal hormone preparation that would not induce any alteration in ovarian function, theoretically would be of great advantage. Despite the reported effectiveness of the use of certain oral preparations of relaxin in the treatment of dysmenorrhea, the results of our clinical studies do not bear out these enthusiastic data.

It is our feeling that the data presented by others promulgating the usefulness of relaxin in dysmenorrhea are based primarily upon the pharmacological action of a placebo rather than upon any therapeutic benefit of the drug itself. Indeed, it would have been unusual for the oral administration of such extracts to be effective therapeutically. Ingestion of the protein, aqueous-soluble material must of necessity result in partial or complete breakdown of the active components of the menstruum by the gastric and intestinal proteolytic enzymes. Consequently, the pharmacological activity of relaxin demonstrable in *in vivo* or *in vitro* techniques would not be anticipated when these materials are administered orally, since they are partially or totally inactivated by the digestive enzymes. On the other hand, the parenteral use of relaxin, the same preparation that was ineffective orally, has yielded effects that must be considered satisfactory and appropriate.

The effective use of parenteral relaxin in these patients must be accomplished by the premenstrual injection of the material according to the time schedule already recommended. Premenstrual administration of relaxin must be obtained in order for the drug to be effective. Relaxin so administered has good therapeutic potentials and warrants trial in those women in whom dysmenorrhea is a problem and in whom there is a contraindication to the use of steroidal therapy.

In the selection of patients with dysmenorrhea for treatment with relaxin, we should like to emphasize that one does not include those patients in whom salicylates or the usual APC compounds are found to be effective. A patient with a case of primary intractable dysmenorrhea that fails to respond to the usual mild analgesics presents a challenge to the physician that is doubly pertinent in that such a patient not only experiences discomfort but sustains economic loss through absence from her employment. The use of relaxin in a case of this sort provides the physician with a nonsteroidal preparation that is free of potential adverse effects.

Preliminary *in vitro* studies with relaxin indicate that the drug has both a relaxing effect and an associated subsequent increase in muscular tonicity in progesterone-primed tissue. These results were noted in both the rat uterus and human Fallopian tube. The significance of these data will be presented in a later report. However, one can anticipate theoretically that the use of relaxin in the parturient patient may first induce relaxation followed by contraction.

If relaxation is confined to the lower uterine segment and cervix and contraction to the fundus, one can readily understand and explain the excellent results obtained in precipitating labor by Birnberg and Abitbol,[10] who demonstrated that relaxin, properly administered, may shorten the period of labor.

Summary

Oral relaxin preparations were administered to a series of women with primary intractable dysmenorrhea. In a control study utilizing the double-blind testing technique with placebo drugs, we were unable to show any benefit attributable to orally administered relaxin preparations over and above that obtained with the placebos alone. On the other hand, premenstrual but parenteral administration of relaxin was effective in controlling dysmenorrhea in fifteen of nineteen patients. It was felt that oral administration of relaxin is an ineffectual and inappropriate form of therapy because of the destruction of the protein material by the proteolytic enzymes of the digestive tract. *In vitro* studies with the relaxin product prepared from the ovaries obtained from pregnant sows indicated that this preparation induced muscular relaxation followed by tonic contraction of both the human Fallopian tube and rat uteri obtained from animals previously primed with progesterone. Uteri obtained from rats primed with estrogen responded with relaxation without subsequent increased muscle tonicity.

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EFFECTS OF RELAXIN ON TERM AND PREMATURE LABOR

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Relaxin, the hormone of pregnancy, was first isolated by Hisaw in 1929.[10] It was found to have the following important actions in the pregnant animal: 1) relaxation of the joints and ligaments,[2, 9, 10] (2) inhibition of the myometrial activity,[5, 13, 17] and (3) softening of the uterine cervix.[8, 18] The physiology, biochemistry and pharmacology of the drug, animal experiments, as well as clinical tests of its inhibitory action on the premature labors, have been described previously.[1, 4, 6, 7, 10-12] The purpose of the present study is to evaluate the claim that relaxin is capable of softening the human pregnant cervix at term.

It is a common experience that the "unripe" cervix makes the induction of labor with Pitocin* a very difficult if not impossible task. If the unripe cervix is treated with relaxin it should become softer, thus making the induction of labor easier. In order to prove this contention, Releasin† was given to a group of patients with unripe cervices at or near term prior to Pitocin induction of labor. The outcome of this group treatment was compared with the results of induction of labor with Pitocin in another group of patients serving as a control. A significantly larger number of successful inductions in the relaxin-treated group should constitute indirect evidence of the softening effect of the hormone on the cervix.

Material and Methods

The study was conducted on 62 pregnant women with unripe cervices at or near term, for whom indicated or elective induction of labor was contemplated. The only criterion for selection was the condition of the cervix. The characteristics on which we based our definition of unripe cervix were as follows: firm consistency, no effacement, no dilatation, and posterior location. The dosage of relaxin varied in the beginning, since the effective amount of this drug in humans has not been established. After several trials, the following scheme was adopted. On the night before the induction, 2 cc. of relaxin was given intramuscularly. On the following morning 6 cc. of the drug was given by intravenous continuous drip in 5 per cent G/W. The rate of infusion was 60 drops/min. Induction of labor was started 2 to 4 hours after the completion of relaxin infusion. One cc. of Pitocin in 1000 cc. of G/W was used initially. If labor was not established, another trial of induction with the same amount of Pitocin was repeated on the following morning. If delivery did not follow after the above regimen, the case was considered a failure.

Thirty-five pregnant women serving as controls were selected among the prenatal clinic patients. The same criteria for the selection as in the study group were observed; the treatment varied only by substitution of placebo for relaxin. Patients who went into labor spontaneously or whose membranes ruptured before the initiation of the Pitocin induction were excluded from the study. No amniotomy was performed unless labor was well established. The above measures served to eliminate other variables, so that the study could be limited to evaluation of relaxin and Pitocin only.

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* Parke, Davis & Company, Detroit, Mich.

† Warner-Chilcott Laboratories, Morris Plains, N. J.

The study group consisted of 7 primiparas, 21 para i, 19 para ii, and 15 para iii or more, for a total of 62 patients. The youngest patient was 17; the oldest, 40. Indications for induction included toxemia of pregnancy, diabetes, Rh sensitization, and retention of a dead fetus *in utero*. There were also several patients under investigation for metabolic changes in anemia of pregnancy in whom it was necessary to perform induction for study purposes. The 35 patients of the control group included 1 primipara, 16 para i, 10 para ii, and 8 para iii or more.

Results

Forty-three (71.5 per cent) patients primed with relaxin delivered after 2 bottles or less of Pitocin. The smallest amount of Pitocin used was 0.5 cc.; the largest, 2 cc. The shortest time elapsing between onset of Pitocin infusion and full dilatation was 50 min.; the longest, 56 hours. Attempt at induction failed in 19 patients (28.5 per cent).

The results in the control group were as follows. There were 8 successfully induced deliveries (22.8 per cent) and 27 failures (77.2 per cent). The shortest time between start of Pitocin and full dilatation was 8 hours; the longest time, 29 hours. Softening of the cervix, as determined on vaginal examination, was noted in all cases treated with relaxin. In no case, however, was increased effacement or dilatation noted. There was 1 fetal death in this series, but the cause of death was clearly demonstrated to be unrelated to the procedure.

Three cases of missed labor with retained products of conception showed a striking response to relaxin-Pitocin treatment. Some of them had had previous unsuccessful attempts at stimulation of labor. The surprisingly uniform effective action of the compound in these cases in which induction of labor is notoriously difficult merits special consideration.

The transient nature of the relaxin effect was observed in some of our cases; the cervix became soft after relaxin infusion, but was firm again the following morning. This effect also is in keeping with the information gained from animal experiments in which it was established that the peak of action occurs in 6 hours, the tissue reverting in 12 hours to the pretreatment state.[11] Very little untoward effect of the drug was noted. One patient developed an urticaria-type rash that responded promptly to antihistaminics. Several other patients complained of chills. The apparent harmlessness of the compound has been reported by previous investigators.[4, 6, 12, 15]

Discussion

The concept of the softening effect of relaxin on the uterine cervix is not new. This action has been observed in animals and humans. Graham and Dracy[8] sed relaxin with estrogen-treated nonestrous cows in which it was necessary to erform dilatation of the cervix to make possible the experimental artificial ansfer of ova. Mechanical dilatation of the cervix cannot be done in the onestrous animal; it becomes easy after treatment with estrogen and relaxin. arrow[18] showed that relaxin administration made it possible to introduce rge metal dilators into the cervices of estrogen-primed castrated sows. He as able also to demonstrate by histochemical methods the increased depolymerization and water solubility of the glycoproteins of the ground substance the relaxin-treated cervix. Similar changes occur in the connective tissue f the pelvic joints and ligaments of pregnant or relaxin-treated animals. olsome *et al.*,[6] who were using relaxin to inhibit the uterine contractions in remature labor, observed that, in cases where the desired arrest of uterine ction did not take place, the labors had an easy, rapid, and occasionally painess character. Eichner,[4] in 1955, reported inconclusive results of his trials of nduction of labor with a relaxin-Pitocin mixture. Evaluation of his study ppears difficult, however, because his material was not limited to cases with nly "difficult" cervices, and there were no normal controls. Eichner states, owever, that in cases where therapeutic abortion had to be performed in advanced stages of pregnancy, treatment with relaxin greatly facilitated the ervical dilatation and evacuation of the products of gestation.

The mechanism of initiation of labor is very complex and, for the most part, bscure. Reynolds[16] cites at least fifteen physiological factors in the term terus that are associated with the onset of labor. In preparation for labor, the ervix usually softens, and the myometrium becomes more irritable and sensiive to oxytocic stimuli. Whether these two events are a part of the same echanism or occur independently is a matter of conjecture. Experience hows that the condition of the cervix is a good index to the receptivity of the terine musculature. On the other hand, the cases that failed in our study ttest to the fact that the cervical changes alone are not sufficient to trigger he mechanism of labor. At any rate, it is apparent that relaxin does not intere with the oxytocic effect of Pitocin. This fact was also observed in the *n vitro* experiments where spontaneous contractions of the uterine muscle trips inhibited with relaxin could be restored with Pitocin.[17] Our experiment rovides statistically valid proof for the effective action of relaxin on the cervix t term. The compound made possible the induction of labor in many cases hat otherwise would have necessitated sectioning or would have required any days of repeated courses of stimulation, with loss of precious time and ith increased danger to both mother and fetus. This fact has considerable ractical importance, since termination of pregnancy near term becomes deirable in a number of conditions including toxemia, diabetes, Rh sensitization, r retained dead fetus *in utero*. In such cases an unprepared cervix is often a erious obstacle. The success obtained in treatment of missed labors appears specially valuable in view of the well-known fact that the medical induction f these cases brings poor results.

We are aware of the shortcomings of our study, in which the action of relaxin as tried by crude clinical methods. The number of cases tested also is not sufficiently large to permit definite conclusions. The investigation would be much more meaningful if clinical evidence of activity of the compound could be paralleled by demonstration of rising blood levels of the hormone. The bioassay methods[3, 14] available at present require excessively large amounts of blood, however, and the accuracy of the test is seriously questioned by some authorities (R. L. Kroc, personal communication).

A more detailed analysis of our results and an evaluation of the possible role of various factors, such as age of the patient, parity, size of the infant, or station of the presenting part, also are difficult in view of the relatively small number of cases.

At present, a study of histochemical changes in the cervix of the relaxin-treated patients is being conducted in our institution. This work should furnish a more objective evidence of the biological action of the hormone to support our clinical impression.

Summary

Pitocin was successful in inducing labor in 71.5 per cent of patients who were primed with relaxin because of an unfavorable cervical condition. In a control group, only 22.8 per cent were successful.

It has been clinically established that relaxin softens the human cervix at term.

This softer cervix apparently is associated with an increased response of the uterus to oxytocin.

In cases that failed to respond, the failure may be due to factors in the initiation of labor, which as yet are not understood.

Relaxin is a useful adjuvant for medical induction of labor.

Further studies are necessary to achieve better understanding of the mechanism of action and optimum dosage of relaxin.

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THE USE OF CERVILAXIN IN TERM LABOR

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Our interest in nonsteroidal ovarian hormones was stimulated by reports of their effectiveness in preventing premature labor. In many instances, however, personal experience has indicated that use of the hormone has been followed by prompt delivery rather than by suspension of labor. In addition, a marked softening of the cervix in these patients also was noted. These observations prompted a consideration of the use of the hormone in expediting and facilitating labor. Zarrow *et al.*[1] and Graham and Dracy[2] have reported that administration of relaxin containing ovarian extracts to gilts and cows resulted in a softening of the cervix.

The hormone used in these studies was extracted from pregnant sow ovaries and purified. The method of purification employed appears to result in a concentration of the cervical softening factor and, thus, has been called Cervilaxin* in order to distinguish it from other ovarian extracts that contain a preponderance of guinea pig symphyseal pubis relaxing factor and a relatively low concentration of cervical softening factor. The possible existence of a complex of protein hormones in the ovary will be discussed in a subsequent publication.

An extensive clinical study involving more than two hundred patients resulted in the formulation of a procedure for administration of the hormone and the conditions under which maximum therapeutic effect could be attained.

TABLE 1 presents the necessary criteria for the successful use of Cervilaxin and also the method of administration of the hormone in conjunction with oxytocin. It must be stressed at this point that these recommended criteria are essential for the successful use of the hormone; otherwise, erratic results and failures will ensue. The normal nonpregnant cervix is 2 to 3 cm. long and about 1.4 cm. wide. During pregnancy the Braxton Hicks contractions gradually shorten the cervix and thin it out. The effacement or thinning-out process varies with the individual and gravidity. Effacement before the onset of labor occurs much more frequently in primipara than in multipara. Interpretation of the degree of effacement will vary among obstetricians. If one assumes the average thickness of the cervix to be approximately 1.5 cm., then 4 mm. would indicate 75 per cent effacement. These are the measurements used to establish our criteria. It is useful for the attending obstetrician to know the thickness of the patient's cervix before the onset of labor.

The results obtained with Cervilaxin in expediting delivery were striking. TABLE 2 presents a summary of the length of labor in 56 patients receiving oxytocin alone, and in 188 patients treated with Cervilaxin and oxytocin as described. These groups include both primipara and multipara, all of whom satisfied the initial criteria of 75 per cent effacement and at least 3-cm. dilatation of the cervix. Duration of labor in the Cervilaxin-treated group was

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* Supplied by National Drug Co., Philadelphia, Pa.

TABLE 1

USE OF CERVILAXIN IN TERM LABOR

(1) Criteria to be satisfied before Cervilaxin treatment is initiated.
  (a) The cervix should be at least 75 per cent effaced and not less than 3 cm. dilated.
  (b) If the membranes are intact, they should be ruptured artificially.
  (c) Uterine contractions should be strong and regular.
  (d) Four to 5 minims (2.5 to 3.1 I.U.) of oxytocin in 1000 ml. of 5-per cent glucose in water is infused intravenously, starting at a rate of approximately 30 drops/min. The oxytocin must continue to run until after the delivery of the baby and placenta.
  Note: The rate and quantity of infusion must be varied according to the intensity of the contractions.
(2) Cervilaxin treatment.
  When the above criteria are present or established by oxytocin, then 20 mg. Cervilaxin in 10 ml. of 5 per cent glucose in water is injected into the clysis tubing immediately above the drip bulb.

TABLE 2

LENGTH OF LABOR IN PATIENTS TREATED WITH OXYTOCIN AND THOSE TREATED WITH CERVILAXIN PLUS OXYTOCIN, UTILIZING THE RECOMMENDED CRITERIA

Number of cases	Cervical dilation (cm.)	Treatment	Average length of labor
Primiparas			
14	3	Oxy.*	3 hr. 50 min.
6	4	Oxy.	3 hr. 55 min.
6	5	Oxy.	2 hr. 10 min.
			Average 3 hr. 18 min.
100	3	Cerv.† + oxy.	1 hr. 20 min.
17	4	Cerv. + oxy.	1 hr. 10 min.
3	5	Cerv. + oxy.	0 hr. 50 min.
			Average 1 hr. 7 min.
Multiparas			
19	3	Oxy.	1 hr. 55 min.
11	4	Oxy.	1 hr. 35 min.
			Average 1 hr. 45 min.
64	3	Cerv. + oxy.	0 hr. 26 min.
4	4	Cerv. + oxy.	0 hr. 20 min.
			Average 23 min.

* Oxy. = oxytocin.
† Cerv. = Cervilaxin.

reduced by about two thirds when compared with that of patients treated with oxytocin alone. Similar results in a smaller group of patients have been reported by Pou de Santiago and Stabile.[3]

A marked softening of the cervix promptly follows infusion of Cervilaxin.

This is of special interest, as it provides for an easier descent and less molding of the fetal head. About 1 hour after the infusion of the hormone, a pronounced softening of the perineum is evident, which persists for 5 to 6 hours. This phenomenon also has been noted by other investigators.[3]

The hormone was next used in the induction of labor. A series of 18 cases was induced by stripping and rupturing the membranes, followed by administration of oxytocin until the cervix was 3 cm. dilated and 75 per cent effaced. At this point Cervilaxin was administered as described (TABLE 3). Once again the marked reduction in the duration of labor is evident when compared to a control series receiving oxytocin alone.

The procedure utilized to obtain the therapeutic results described above was arrived at after considerable trial and error. A presentation of the preliminary work that led to the adoption of the present method (TABLE 1) is described below.

A summary of early observations on the effect of Cervilaxin in normal labor is presented in TABLE 4. In this series, Cervilaxin was administered without oxytocin or regard to the stage of labor, effacement, or dilatation of the cervix. No over-all shortening of the duration of labor was noted and, in several cases, there was cessation of labor, an observation that prompted the employment of oxytocin in subsequent studies. It is significant, however, that in 70 per

TABLE 3
INDUCTION OF LABOR WITH CERVILAXIN-OXYTOCIN

Patient*	Gravida	Length of labor after Cervilaxin (min.)
L. S.	1	70
C. B.	1	55
A. M.	1	75
D. D.	1	60
M. C.	1	50
G. C.	1	80
T. D.	1	40
E. S.	1	50
R. P.	1	70
M. Z.	1	60
		Average 61
B. C.	2	40
E. M.	2	23
G. W.	2	35
R. W.	2	25
B. E.	2	25
B. K.	2	20
E. N.	2	40
J. B.	3	20
		Average 29

* These patients were all at term and had elective induction accomplished by stripping and rupturing of the membranes and infusion with oxytocin until the cervix was 75 per cent effaced and 3 cm. dilated. Cervilaxin was then administered intravenously as described.

ent of these cases the Cervilaxin administration caused cervical and perineal oftening.

In the next series (TABLE 5), oxytocin in conjunction with Cervilaxin was ised. However, the hormones were administered before dilatation and effacenent had reached the stage previously mentioned, that is, 3 cm. dilatation of he cervix and 75 per cent effacement. In this series, a variability of response vas observed, which indicated the unpredictability of successful use if the ormone is administered before the established criteria for effectiveness have een reached.

A summary of early experiences with Cervilaxin and oxytocin in inducing abor is presented in TABLE 6. In these cases the membranes were stripped rom the cervix and ruptured. The cervix was thick and, in most cases, there vas no dilatation. Hormone administration was started immediately without awaiting the necessary effacement and dilatation. In all instances, the primipara manifested an unsatisfactory course of labor, with irregular contractions. Effacement was never complete, even when full dilatation was present. In the multipara, labor was also poor; the cervix dilated slowly, and effacement was

TABLE 4

USE OF CERVILAXIN ALONE IN NORMAL LABOR (RECOMMENDED CRITERIA NOT EMPLOYED): 30 CASES

Effect on shortening of labor	Cervical and perineal softening
30 Cases (20 primipara; 10 multipara)	
Not effective	
(a) Labor stopped at 6–7 cm. dilation for 4–6 hr. Then resumed spontaneously.	
6 primipara	6 positve
3 multipara	3 positive
(b) Labor stopped completely. Delivered 48–72 hours later.	
2 primipara	2 positive
1 multipara	1 positive
(c) Labor not effected.	
12 primipara	8 positive 4 neg.
6 multipara	4 positive 2 neg.
Effective None	24 positive

TABLE 5

CERVILAXIN AND OXYTOCIN SIMULTANEOUSLY ADMINISTERED BEFORE 3-CM. DILATATION AND 75 PER CENT EFFACEMENT OF THE CERVIX

Number of cases	Parity	Cervical dilatation	Effacement	Average time of labor
45	Primi	1–2 cm.	<50%	12.3 hr.
15	Primi	1–2 cm.	<50%	2 hr.
28	Multi	1–2 cm.	<50%	1 hr. 45 min.
12	Multi	1–2 cm.	<50%	30 min.
		Thick—less than 50 per cent effaced		

unsatisfactory. Many of these cases delivered with thick cervices. Even though there was no apparent shortening of the duration of labor, 80 per cent of the patients exhibited marked cervical softening. In contrast to these poor results were the excellent results obtained with our present regimen (TABLE 3).

At this time it is desirable to comment upon a significant use for Cervilaxin, namely, its employment in cases of true or borderline disproportion. When properly employed, it provides a rapid and satisfactory answer as to whether safe vaginal delivery can be accomplished. In a group of such patients, the therapy produced the usual cervical softening and complete cervical dilatation, followed by prompt descent of the fetal head when vaginal delivery was possible. If the fetal position was either occipitoposterior or in deep transverse arrest, then correction of the position by forceps rotation would permit vaginal delivery, provided no cephalopelvic disproportion was present. This could be determined after correction of the malposition. If, after one hour, no further descent was noted, cesarean section was performed (TABLE 7).

TABLE 6

INDUCTION OF LABOR (CERVILAXIN AND OXYTOCIN SIMULTANEOUSLY ADMINISTERED BEFORE 3-CM. DILATATION AND 75 PER CENT EFFACEMENT)

Number of cases	Gravida	Average time of labor
33	Primi	14.6 hr.
7	Primi	10.4 hr.
55	Multi	5.4 hr.
5	Multi	2.8 hr.

TABLE 7

CERVILAXIN INFUSION AS A PROGNOSTIC AID IN DYSTOCIA

Case	Parity	Condition prior to Cervilaxin-oxytocin	With Cervilaxin-oxytocin full dilation reached within:	Then no progress of labor, and cesarean section performed after:
1	0	Good labor, cervix effaced, 5 cm. dilatated; station minus 2; membranes intact	Thirty min.; station 0; membranes ruptured	One hr. 30 min.; breech (9 lb.)
2	0	Good labor, cervix effaced, 4 cm. dilatated; station minus 1; membranes ruptured	Ten min.; station 0	Two hr.; 7 lb., 2 oz.; cephalopelvic disproportion
3	2	Good labor, cervix 3 cm. dilatated, 75% effaced; membranes ruptured; station minus 3	Thirty min.; station minus 3	Three hr.; 8 lb., 1 oz.; cephalopelvic disproportion
4	0	Good labor, cervix 3 cm. dilatated, 75% effaced; station minus 1; membranes intact	One hr. 45 min.; station minus 1; membranes ruptured	Six hr. 30 min.; 8 lbs.; cephalopelvic disproportion
5	0	Moderate labor, cervix 2 cm. dilatated, 75% effaced; membranes ruptured; station minus 2	Two hr.; station minus 2; cervix 5 cm. dilatated	Nine hr.; 8 lb. 9 oz.; cephalopelvic disproportion

Cases of prolonged or poor labor that, after oxytocin, Demerol, and scopolamine therapy, still exhibited poor contractions and incomplete dilatation of the cervix could be brought to successful termination and delivery with Cervilaxin. Sixteen primiparas, from other obstetrical services, who had been in labor from 12 to 24 hours and showed poor uterine contractions, with arrested cervical dilatation, were brought to our attention. Cervilaxin therapy was recommended and instituted. Uneventful delivery occurred within 2½ hours in 14 of these patients, the other 2 going to cesarean section—1 for compound presentation (hand and head) and the other because no further dilatation of the cervix occurred (TABLE 8).

We have utilized the ability of Cervilaxin to produce a softening of the cervix in the delivery of double footlings. This type of presentation results in a high degree of fetal mortality, and it has been our practice to deliver all such cases, regardless of parity, by cesarean section. This obstetrical difficulty is produced by the progression of the feet and body in spite of poor dilatation of the cervix, with subsequent prevention of delivery of the head. We were able to deliver successfully a presentation of this sort by administration of oxytocin and Cervilaxin, with consequent softening of the cervix to permit passage of the fetal head with ease.

In conclusion, one may categorize the results obtained from the use of Cervilaxin in parturition as follows:

(1) Full dilatation and softening of the cervix are obtained quickly with consequent rapid delivery in normal cases.

(2) Because of the rapidity with which full dilatation is obtained, a diagnosis

TABLE 8

EFFECT OF CERVILAXIN ON PROLONGED LABOR

Patient	Gravida	Therapy	Cervical dilatation (cm.)	Duration of cervical arrest (hr.)	Time in labor up to Cervilaxin (hr.)	Time to delivery after Cervilaxin
1	1	Pit. + Dem.*	6	4	19	2 hr. 10 min.
2	1	Pit. + Dem.*	6	6	16	2 hr. 0 min.
3	1	Pit. + Dem.*	8	6	23	2 hr. 40 min.
4	1	Pit. + Dem.*	6	3	17	2 hr. 0 min.
5	1	Pit. + Dem.*	5	6	20	2 hr. 5 min.
6	1	Pit. + Dem.*	7	3	17	Cesarean (prolapsed hand)
7	1	Pit. + Dem.*	7	5	19	2 hr. 30 min.
8	1	Pit. + Dem.*	7	5	21	2 hr. 0 min.
9	1	Pit. + Dem.*	7	5	34	1 hr. 50 min.
10	1	Pit. + Dem.*	8	5	15	2 hr. 0 min.
11	1	Pit. + Dem.*	7	3	15	2 hr. 15 min.
12	1	Pit. + Dem.*	6	3½	15	2 hr. 10 min.
13	1	Pit. + Dem.*	6	4	12	2 hr. 15 min.
14	1	Pit. + Dem.*	6	4	16	1 hr. 45 min.
15	1	Pit. + Dem.*	5	9	18	Cesarean section
16	1	Pit. + Dem.*	7	4	22	2 hr. 0 min.

* Pitocin + Demerol.

of cephalopelvic disproportion or abnormal position can be made rapidly, thus avoiding prolonged labor with subsequent damage to mother and infant.

(3) Cervilaxin has been found to overcome the difficulties associated with cervical dystocia problems.

(4) The softening of the cervix after administration of Cervilaxin is suggested to be of value in the management of footling breeches.

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THE EFFECTS OF RELAXIN ON TERM AND PREMATURE LABOR

By Eduard Eichner, Irwin Herman, Leonard Kritzer, Gerald M. Platock, and Lee Rubinstein

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This paper covers our personal experiences during the past seven years. The preparations of relaxin used have varied in titratable potency, in solvents, and in additives. Two manufacturers have prepared these products, which are now made up in physiological saline, are standardized to contain 20 mg. of relaxin standard per cubic centimeter, and are available on the open market.* This material should be refrigerated, but never frozen, and may be used intramuscularly or intravenously with the usual precautions.

Our original experiences were reported in 1955, and this work was repeated with the newer materials. When we became familiar with the similarities and differences of these products, the work was extended on a comparative basis, and included attempts to stop premature labor, to stop or modify term labor, and to expedite the induction of labor. Where feasible, tokodynomometric (TKD) or oscillographic studies were made. The effect on cervical softening and effacement was studied further clinically on a group of women in the thirty-eighth to fortieth weeks of gestation. None were in labor, and none delivered within 72 hours of their respective study.

The greatest difficulty in the evaluation of the effects of drugs on labor is the identification of labor. Even cursory perusal of the obstetric literature makes manifest the multiple interpretations of the term. Tokodynomometric tracings may not be too helpful in early labor. Patients in our series who were in labor were active and making progress before medication was initiated. Cervical dilatation was increasing, and descent of the presenting part was invariably present. Occasionally we erred in both directions in determining the actual presence of labor. When labor was present according to our standards, we were unable to distinguish premature from term labor. The only clinical difference noted was the ability of the premature infant to be born through cervical dilatation incompatible with term delivery.

Modification of Labor

Through trial and error, the following routine was established. An intravenous infusion of 5 per cent dextrose or levulose in water was started. Relaxin was added, and the flow was adjusted so that 3 to 5 mg. of relaxin per minute was administered to the patient. When labor was very active, a single massive intravenous injection of 100 to 200 mg. might be given at the start. Invariably this injection produced an immediate effect (FIGURE 1). If response to the original rate of infusion was not evident in 10 to 15 min., it was increased to

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* Releasin, the Warner-Chilcott Laboratories (Morris Plains, N. J.) brand of relaxin, contains 20 mg. of relaxin standard (3000 g.p.u.) per cc., and is available in 1 cc. ampoules; Cervilaxin, the National Drug Company (Philadelphia, Pa.) brand of relaxin, contains 20 mg. of relaxin standard (4000 g.p.u.) per cc., and is available in 2 cc. vials. The units are not identical.

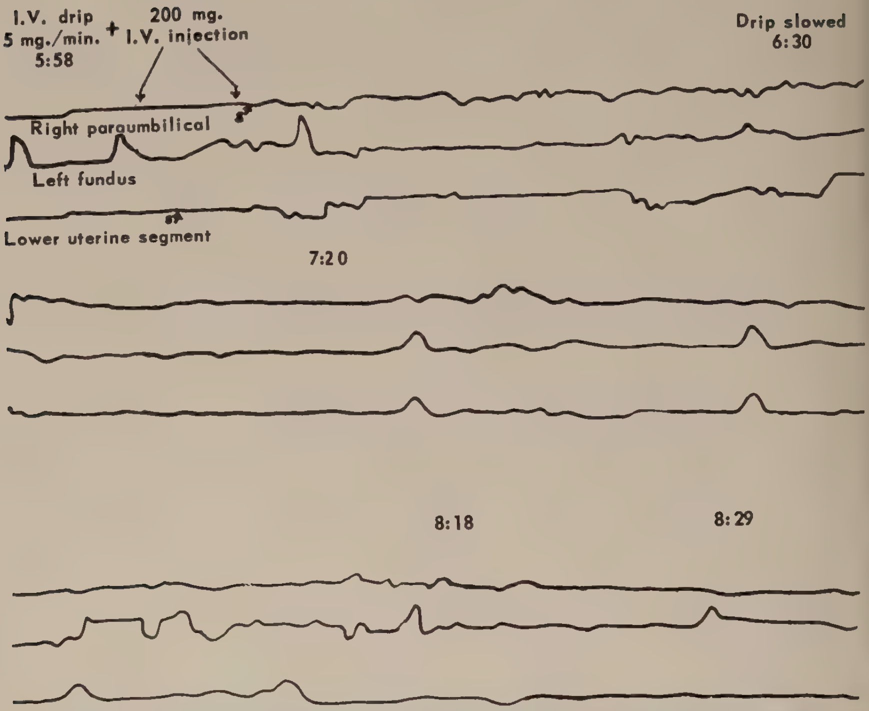

FIGURE 1. U.H. No. 235-819, triplets in premature labor at 25 weeks' gestation. The center line of the upper portion of the tripolar TKD tracing records first, and shows contractions at 2-min. intervals, despite relaxin drip at 5 mg./min. The patient received 200 mg. relaxin (National Drug Company) intravenously, and there was an immediate disorganization of the ensuing contractions. Regular contractions, which did not persist, recurred about 1 hour later. Medication was maintained for approximately 24 hours, and the patient did not deliver until her thirty-fifth week.

approximately 6 to 8 mg./min., and then, if necessary, to 10 to 12 mg./min. If contractions had not become appreciably modified by this dosage within 15 min., medication was discontinued. If contractions became irregular and stopped, the effective rate was continued for at least 1 hour before it was reduced (FIGURE 2). Effective control was practically never maintained at less than 2 mg./min., and usually had to be kept at the 4- to 6-mg./min. level. Maintenance dosage was continued for from 4 to 24 hours. Concomitant intramuscular dosage was begun after 4 hours of maintenance, but the actual schedule varied with the patient's needs.

Intravenous medication was repeated whenever intramuscular dosage did not hold. If the interim was less than 4 to 6 hours, relief could be obtained by the maintenance schedule (FIGURE 3). Should the interval exceed this arbitrary time limit, medication was started as if the patient had never been treated, but the new initial dose was the same as the former maintenance one. We have not tested patients for sensitivity before retreatment, and have seen no

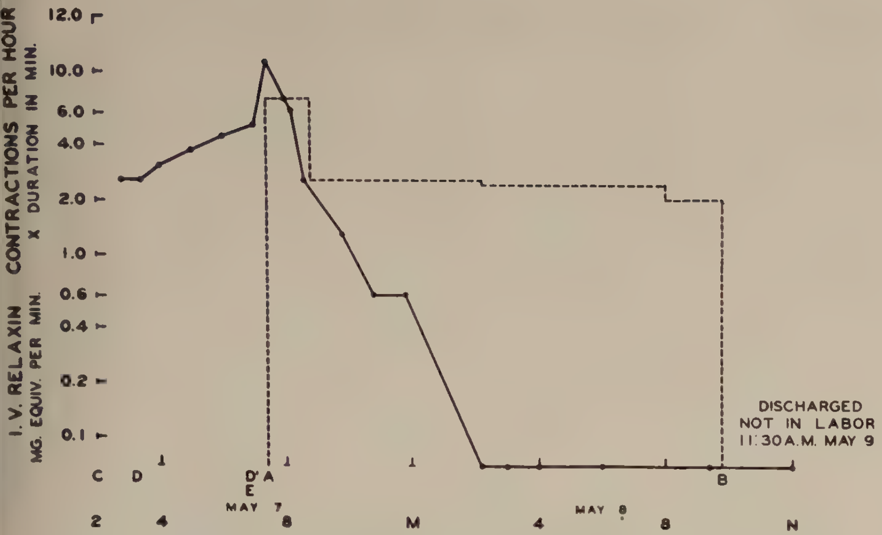

FIGURE 2. Semilogarithmic graph demonstrating the effect of relaxin on early labor—J.S. No. 38695 (M.S.), 28 weeks' gestation, admitted in early active labor. Symbols: -------- = dosage of intravenous injection of relaxin; ——— = strength of labor (number of contractions per hour multiplied by the duration of contractions in minutes); A = start of relaxin therapy; B = discontinuation of therapy; C = stilbestrol given 25 mg. every 15 min. for 8 doses; D and D′ = Demerol, 100 mg.; E = secobarbital, 200 mg. Previously published in the *American Journal of Obstetrics and Gynecology*;[1] reproduced by permission of The C. V. Mosby Company, St. Louis, Mo.

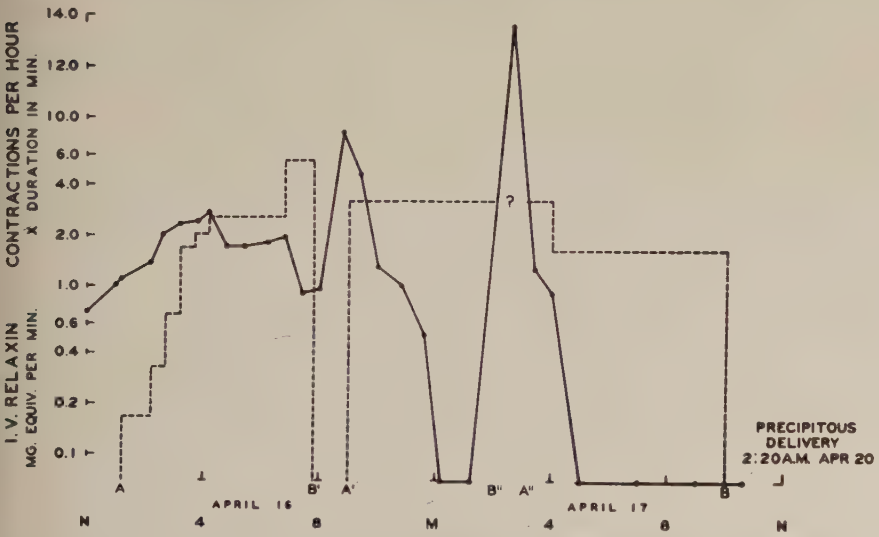

FIGURE 3. Semilogarithmic graph demonstrating the effect of relaxin on early labor—M. N., No. 15333 (M.S.), 31 weeks' gestation, premature rupture of membranes (5 days). Symbols: -------- = dosage of intravenous injection of relaxin; ——— = strength of labor (number of contractions per hour multiplied by the duration of contractions in minutes); A, A′, and A″ = respective starts of relaxin therapy; B, B′, and B″ = respective discontinuations of therapy. Previously published in the *American Journal of Obstetrics and Gynecology*;[1] reproduced by permission of The C. V. Mosby Company, St. Louis, Mo.

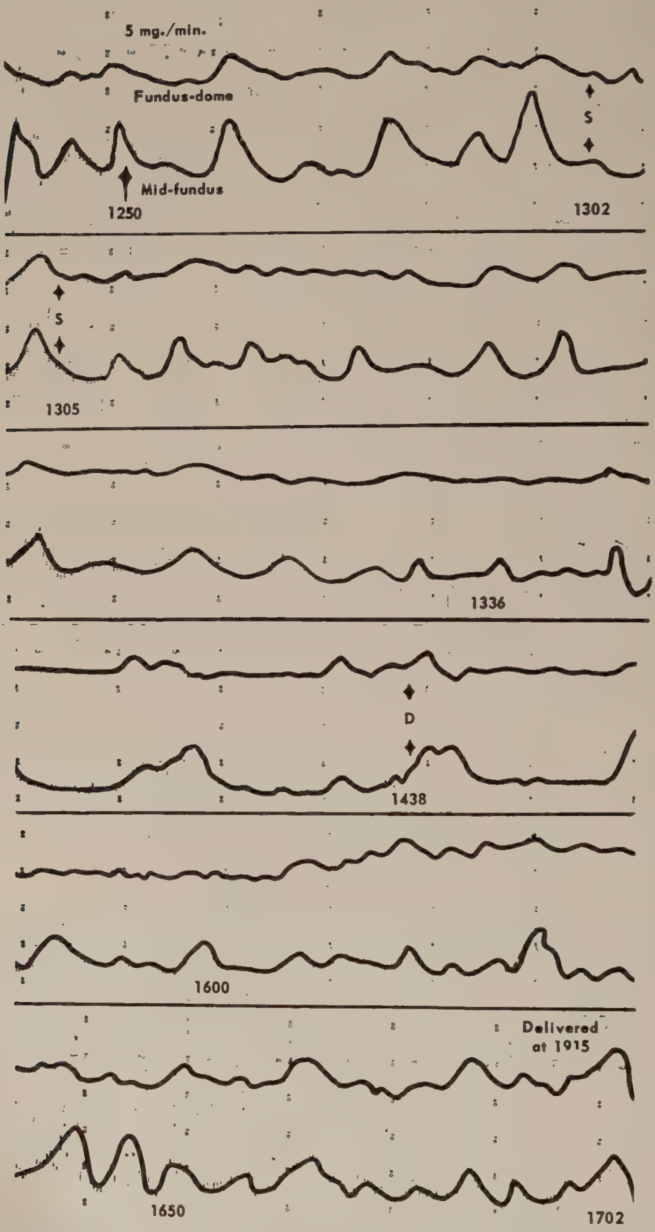

FIGURE 4. U.H., No. 678-214, bipolar TKD tracing of normal term labor. Rapid intravenous injection of relaxin (W.C.), 5 mg./min. at S′ resulted in ineffectual and weak contractions. Cervical dilatation (3 cm.) and descent (station 0) did not change until approximately 1½ hours after treatment was discontinued at D. Delivery occurred within 5 hours of the end of treatment, and approximately 3 hours after the return of good contractions. Previously published in the *American Journal of Obstetrics and Gynecology*;[1] reproduced by permission of The C. V. Mosby Company, St. Louis, Mo.

complications. The effect of the initial massive intravenous injection frequently disappeared within 30 to 60 min., even though active medication was continued intravenously.

Disorganized contractions were the first evidence of a successful effect on the inhibition of labor. This was followed by a leveling off of the frequency and duration of the succeeding contractions. Apparently normal but irregular and ineffectual contractions might persist for hours, but labor would not progress (FIGURE 4). In one unsuccessful trial, contractions were almost controlled by the time the cervix reached 7-cm. dilatation. Spontaneous rupture of the membranes occurred here. Precipitate delivery of the first premature twin was followed shortly thereafter by the second infant (FIGURE 5). When therapy was stopped in patients in term labor, regular contraction recurred within the hour, and then labor usually proceeded apace (FIGURE 6).

It was this recurrence of effective and frequently painless labor on ineffectual and inadequate dosage that made us discontinue the use of intramuscular and depot material in the control of premature labor. Many of these patients delivered, unaware that labor had again started until the pressure of the presenting and often visible fetal part was felt. Gelatin and polyvinyl pyrrolidone depot relaxin material was ineffective in our hands. The results obtained in term labor were very similar to those in premature labor, both with the single high potency injection and/or with the prolonged intravenous drip. However, we made no effort to stop term labor completely for any length of time.

We now have treated 41 patients for premature labor in the twenty-fifth to thirty-first weeks of gestation, with living issue in 13. Three cases were previously reported in full. Many also received high dosage of the progestogens

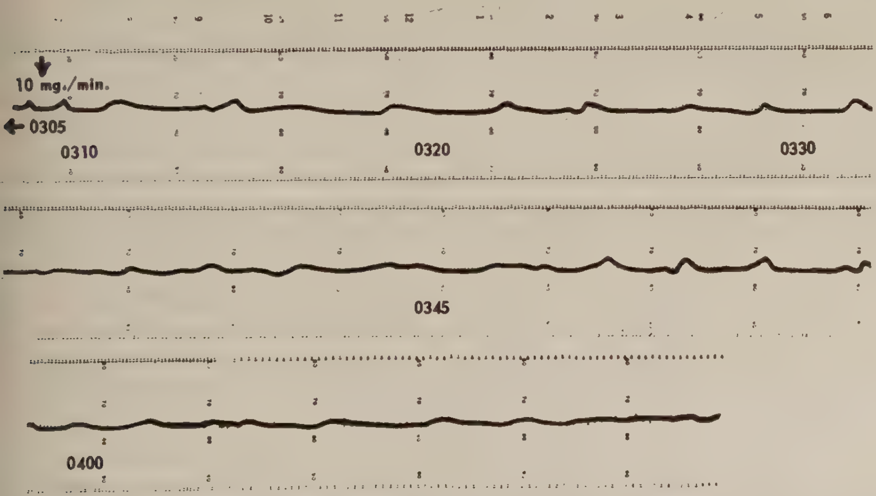

FIGURE 5. U.H., No. 707-640, unipolar TKD tracing of twin pregnancy in active labor. Under intravenous therapy of relaxin (W.C.), 10 mg./min., contractions became farther apart and less strong. However, dilatation continued. The patient ruptured membranes at 7-cm. dilatation at approximately 0350. Labor picked up as the first twin was born shortly after the conclusion of this record.

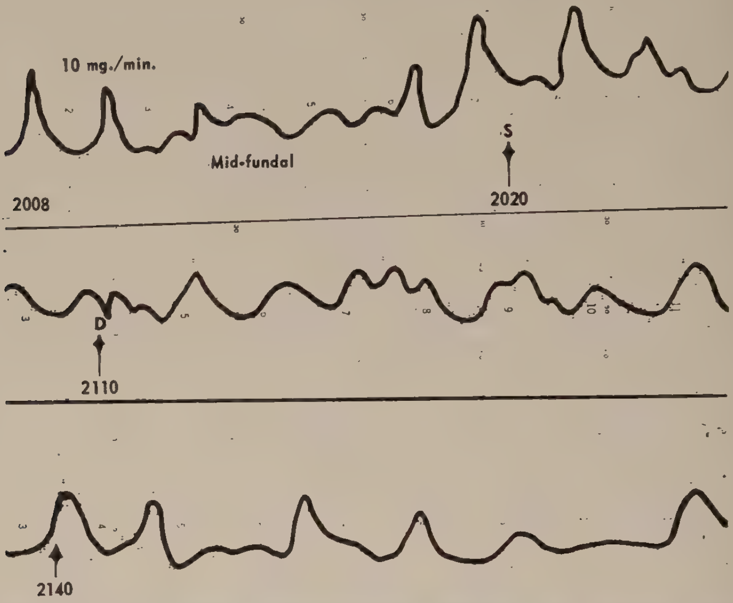

FIGURE 6. U.H., No. 677-559, active term labor, vertex at plus 1, cervix 4 cm. dilated. Relaxin (W.C.) 10 mg./min., was started at S and discontinued at D. Note the recurrence of forceful contractions within ½ hr. Delivery occurred at 0200, slightly less than 5 hours after treatment was stopped. Previously published in the *American Journal of Obstetrics and Gynecology*;[1] reproduced by permission of The C. V. Mosby Company, St. Louis, Mo.

in the prelabor period. Eight additional patients in the premature-labor group experienced precipitate labor and almost painless delivery within 36 hours of the cessation of treatment. Retreatment had not been started because no one was aware of the imminence of recurrent labor. These patients were classified as failures in technique, even though 6 of these infants survived. It was this group of subjects that led us to the conclusion that the cervical effect far outlasts the uterine effect.

Induction of Labor

It was believed that the effect of relaxin on the cervix might be conducive to a state in which induction could be done more expeditiously. It has been noted that relaxin produces a cervical softening effect in many patients. Should this softening remove cervical resistance to the advancing fetus, labor might be accelerated safely. Attempts were made to find the optimal dosage of relaxin that would abet cervical softening without inhibiting the progress of labor. Oxytocin* was eminently successful in some patients who received relaxin, but was

* Syntocinon, the Sandoz Pharmaceuticals (Hanover, N. J.) brand of oxytocin, contains 10 U.S.P. units (10 I.U.) of synthetic oxytocic principle of the posterior lobe of the pituitary gland per cubic centimeter, and is free of vasopressin; Pitocin, the Parke, Davis & Company (Detroit, Mich.) brand of oxytocin, contains 10 U.S.P. units (10 I.U.) of oxytocic principle of the posterior lobe of the pituitary gland per cubic centimeter, and is substantially free of pressor activity.

completely ineffective in others who had an apparently fully ripe cervix. When studied, these patients had inertia-type labors with lack of symmetry and "follow-through" in their contractions. We believe that the uterine effect of relaxin persisted in these subjects.

Relaxin was used intramuscularly or intravenously in dosages ranging up to 100 mg., and oxytocin was used concomitantly or at varying intervals before or after the use of relaxin. Oxytocin also was used intramuscularly or intravenously. Depot materials of relaxin again were ineffective for us. The proper combination of oxytocin and intravenous relaxin varied considerably, but smooth and rapid labor resulted when this combination, different for different patients, was attained. Stripping or rupture of membranes was not done in any patient in this group. In the most frequently successful regime, the patient received 20 mg. of relaxin by intravenous drip at a rate of 1 mg./min. This was followed by dilute intravenous oxytocin at a rate just sufficient to maintain effective contractions. Progressive labor usually was instituted within the first hour, and the majority of patients were delivered in less than 4 hours. In many the dosage of the total intravenous fluids never exceeded 200 cc., and often was under 100 cc. In our hands, providing full dosage was used, there was no difference in the effects of either commercially available relaxin product. Results were less consistent when relaxin dosage was continued through the entire induction. We believe that a myometrial effect was obtained in these patients.

Using the above schedule, 17 patients were induced on the first attempt, 4 on the second. Three had 3 unsuccessful inductions. In 2, these were done on consecutive days. In the third, there was at least 1 week between the second and third trials. Five additional patients, unsuccessful on their first test, were sectioned or induced with rupture of the membranes without further trial. Six were induced with concomitant stripping and/or rupture of the membranes and therefore are excluded. These cases have not been reported previously.

Cervical Softening and Effacement

Because we were unable to predetermine the patient in whom the cervical softening effect would assist an efficient labor, a study was set up in which each member of a group of 40 near-term patients (38 to 40 weeks' gestation) were examined vaginally. Each then received an intravenous injection of 1 cc. of 1 of the 5 unknown solutions—2 samples from each of the manufacturers, plus an unknown blank. Each subject was re-examined vaginally by the original examiner at 1 and 2 hours after the injection. At each examination an estimate was made and noted of the degree of effacement and cervical softening present. These figures were converted to a percentage basis by the senior author, who had neither examined the patients nor seen the coded injection list. The results, tabulated in TABLE 1, usually were apparent within the first hour and had not diminished at the end of the second hour.

In only 6 patients was there any change in effacement. Two received the placebo and 2 received relaxin with the depot additive polyvinyl pyrrolidone. Cervical softening did not change in 21, 16 of whom received solutions of relaxin,

TABLE 1

EFFECT OF RELAXIN ON THE HUMAN CERVIX AT TERM

Experimental product (1 cc. I.V.)	Percentage of effacement		Percentage of cervical softening		Range of percentage of increase in softening
	Start	End	Start	End	
(A) Eleven patients Warner-Chilcott, W 1164-3 P, Lot 002057, physiological saline placebo	0	0	40	40	0
	0	10	20	50	30
	70	70	60	80	20
	30	30	30	50	20
	30	50	20	50	30
	20	20	70	80	10
	20	20	50	60	10
	0	0	30	30	0
	0	0	35	35	0
	0	0	30	30	0
	0	0	10	10	0
(B) Nine patients Warner-Chilcott, W 1164-3, Lot 22347, 20 mg. relaxin standard* (3000 g.p.u.) per cc. in physiological saline	0	0	70	70	0
	0	0	50	50	0
	0	0	40	40	0
	50	50	30	30	0
	0	0	60	60	0
	0	0	20	40	20
	20	20	30	50	20
	10	10	50	70	20
	25	25	75	90	15
(C) Seven patients Warner-Chilcott, W 1164-3, Lot 07547, 20 mg. relaxin standard* (3000 g.p.u.) per cc. in physiological saline	0	25	10	10	0
	0	0	30	50	20
	25	25	50	50	0
	50	50	60	60	0
	25	25	50	50	0
	50	50	60	80	20
	0	0	10	10	0
(D) Seven patients National Drug, Uterine relaxing factor, Lot 5941, 20 mg. relaxin standard* (4000 g.p.u.) per cc. in physiological saline	0	25	50	50	0
	50	50	50	50	0
	0	0	20	30	10
	0	0	30	80	50
	0	0	40	50	10
	0	0	10	60	50
	70	70	30	30	0
(E) Six patients National Drug, Uterine relaxing factor, Lot 5797, 40 mg. relaxin standard* per cc. in polyvinyl pyrrolidone	50	75	50	50	0
	0	0	20	40	20
	25	25	50	50	0
	25	25	60	60	0
	25	50	60	80	20
	50	50	60	80	20

Cervical softening increased in 19, but 6 of these received the placebo. There was no obvious relationship between the degree of effacement at the start of the test and with the final results. There was no difference between the various samples.

Discussion

Clinically, we have been able to interfere with progressive labor at term, and we have been able to stop active premature labor, even starting with a cervical dilatation of 4 cm. However, since 15 to 45 min. may be required for full control, cervical dilatation might have proceeded beyond the "point of no return." Massive primary intravenous therapy should be used if the clinician fears that inhibition of labor may be delayed unreasonably. Premature fetuses can deliver through a cervix dilated not beyond 7 cm. At the present time the cost of the necessary medication is prohibitive. Unless economic conditions are not a factor, this material should be used primarily in those patients without a recurring stimulus to labor, as 750 to 1500 mg. of relaxin is frequently required for the original complete control of labor.

Many patients with Braxton Hicks contractions will lose their uterine irritability without any treatment. It is our opinion that this is the group in which "premature labor" is controlled by intramuscular relaxin, frequently in homeopathic doses. Furthermore, many patients with irritable uteri may proceed into true labor because of the cervical softening effect of relaxin. Thus, inadequate dosage actually may produce the effect it was hoped to avoid.

The cervical softening effect could be important in the induction of labor. Small doses of relaxin did improve our "takes" in oxytocic induction of labor without rupture or stripping of the membranes. However, the effect was inconstant, and the required dosage varied greatly from patient to patient. Despite this, we were not prepared for the surprising results of the experiment on the near-term patients not in labor. The cervical softening effect was very good in the placebo group that received only saline, and there was no significant difference in the results of any of the unknowns. This study is being continued in a modified form.

As a result of our clinical experience, we believe the cervical effect (should we be able to prove its presence) comes first and disappears last on relaxin treatment. Our theory regarding this is a simple one. The effect of relaxin is primarily on the tissue cements of the connective tissue, fibrous and elastic. The cervix is almost 100 per cent connective tissue, and therefore would have an immediate, complete, and persisting effect. The uterine body, on the other hand, is an organ composed of bundles of smooth muscle. As each cell contracts, it transmits its impulse to an adjacent cell through the connecting tissue blending each group of cells into a muscle bundle. A very large amount of relaxin is required to affect these connective tissue cells sufficiently to cause them to lose all cohesiveness. Otherwise, some residuum of tensile power persists, and this is sufficient to transmit impulses from one muscle cell to the next. Since it is required only that the cervix become fully elastic to be efficient in labor, lesser amounts are required here.

In an occasionally supersensitive patient, average amounts of relaxin may so interfere with the normal contraction waves that even forceful contractions produced by oxytocin are ineffectual. We believe that the foregoing adequately explains the apparent discrepancies in varied reports on relaxin, as well as case reports of the patients treated successfully or unsuccessfully on the varied relaxin routines. The key to the final average and feasible dosage has not yet been found.

Summary and Conclusions

Solutions of relaxin, varying in potency and composition, were injected intramuscularly and intravenously into pregnant women in the third and late second trimesters of pregnancy. Injections were given to modify or stop premature and term labors, to expedite the induction of premature and term labor, and to test the cervical softening effect of various solutions of relaxin on pregnant women at term, but not in labor. The techniques of administration and the clinical findings are reported.

Relaxin will modify term labor and can stop premature labor if given in adequate dosage. Ineffectual administration or inadequate dosage will enhance rather than inhibit labor and delivery. The proper combination of relaxin and oxytocin, sometimes difficult to determine, will expedite the induction of premature and term labors. The cervical softening effect occurs first and outlasts the uterine effect. However, the experimental group of women not in labor suggests that this may be the result of some unidentified nonspecific substance in the relaxin solution, and not the result of the pure relaxin complex.

Reference

Eichner, E., C. Waltner, M. Goodman & S. Post. 1956. Relaxin, the third ovarian hormone; its experimental use in women. Am. J. Obstet. Gynecol. **71:** 1035–1048.

DISCUSSION: PART IX

Daniel Abramson (*Boston Lying-in Hospital, Harvard Medical School Boston, Mass.*): To discuss in detail all of these well-presented papers on the clinical aspects of relaxin adequately would require infinitely more space than is available; therefore I shall touch only on the more important points, and as briefly as possible.

I think we should realize at the start that actually we are dealing with three different commercially prepared products whose only chemical relation is that they supposedly contain varying percentages of relaxin. However, as to two of these our assumption may not be correct. In spite of this, claims have been made that all three products produce results more or less comparable clinically.

I am convinced that Releasin is as pure a product of the corpus luteum of pregnant sow ovaries as is obtainable. The assay of this extract is based on the fact that one guinea pig unit administered subcutaneously is sufficient to relax, within 6 to 8 hours, the symphysis pubis of a castrated guinea pig previously brought into artificial estrus.

Lutrexin, I believe, is assayed on uterine muscle, so that 0.002 mg. given intravenously causes relaxation of uterine muscle. Though assayed intravenously, in humans it is administered only orally; when assayed by guinea pig symphysis test, it has been found to possess one thirtieth to one fiftieth the activity of Releasin. Rezek[7] in an earlier publication has stated that Lutrexin had no effect on the symphysis pubis of the guinea pig (see discussion by Bryant, below).

As for the newest of the three products, namely Cervilaxin, there is no indication regarding the method of assay or the nature of its active principle. When assayed by means of the guinea pig symphysis test it is reported to contain one fiftieth to one eightieth the activity of Releasin. It is administered I.V. in humans and, to my knowledge, has no effect on the symphysis pubis of the guinea pig (see discussion by Kleinberg, below).

If we are to continue to compare the clinical effects of these three substances, I hope some effort soon will be made to establish a comparable standard of assay either in regard to relaxin effect or in regard to its respective active principle, should it be known.

In the early commercial preparation of relaxin, the more purified preparation lost some of its effect; therefore, in the case of Lutrexin and Cervilaxin, other ovarian factors may be contributing to their activity.

Preceding papers have dealt with the effects of these three substances in premature and term labor, and in the treatment of dysmenorrhea.

Let us first consider what has been said about premature labor. Rezek's experiences with Lutrexin administered orally are truly remarkable, particularly since his criteria of labor are somewhat different from those used by Stone, Eichner, and myself. Our studies, in which Releasin was used, indicate that once the cervix begins to dilate, labor may be slowed temporarily by relaxin, but certainly not stopped. In fact, due to the probable effect of relaxin on the cervix, once it is dilated by 2 cm. or more, relaxin aids in more rapid dilatation and frequently with much less discomfort than might be expected. It is our feeling in this respect that, perhaps soon, we shall look upon the first stage of labor as consisting of two phases, the first beginning when the patient has regular rhythmic and painful contractions resulting in taking up of the cervix the second beginning when the cervix begins to dilate. It is apparent that, in Stone's experience and in mine, relaxin is effective only in this initial phase of the first stage of labor, namely before the cervix begins to dilate. We usually do not administer relaxin in cases of premature labor if the cervix is found to be more than 1 cm. dilated. We are not concerned if the membranes are ruptured, or if the patient has slight bleeding, unless this should become excessive.

One of our most interesting patients is that of a gravida 2, para 0 who, after her first miscarriage, was shown to have a bicornuate uterus. At 23½ weeks of gestational age she began to have slight to moderate vaginal bleeding associated with regular 4 to 5 min. contractions. She was given 1 cc. of Releasin, that is, 3000 g.p.u. I.M. every hour for 4 doses. Her contractions began to disappear after the second injection and, after the fourth injection, they stopped altogether, as did the bleeding. She was carried along on Norlutin from this point on and, at 34½ weeks, she was delivered by cesarean section of a normal infant. At section, a central praevia was found, thus confirming the preoperative diagnosis.

Although my series now number 50 or more patients who entered the hospital in premature labor and were treated with Releasin, I give here the statistics of only the first 40. In two instances labor started just prior to 24 weeks of gestational age, one of these being a patient with a bicornuate uterus. From over-all statistics one is forced to conclude that relaxin does have a place in the treatment of premature labor, but there is no doubt in my mind that selectivity of patients is important.

Administration of relaxin at term for the purpose of conditioning an otherwise unfavorable cervix is gaining in popularity, and the results are moderately good. Although my own series to date is not as large as that of Stone's, I should certainly agree with him and many others that relaxin conditions the cervix sufficiently to allow for more successful induction. Again, time did not permit an analysis of more recent cases.

Maclure[4] has recently reported from Miami, Fla., on fifteen patients all having undelivered stillborn infants. Using Releasin prior to induction he obtained excellent results in eleven instances. In 2 patients, the results were equivocal and there were 2 failures.

Stone's histochemical studies should produce interesting results when they become available. They may provide further correlation of the work of Graham and Dracy,[3] of F. X. Gassner, University of Colorado, Boulder, Colo. (personal communication), and of Zarrow *et al.*[10, 11] to the effect that relaxin produces changes in the connective tissue of the cervix which Perl and Catchpole[5, 6] call a depolymerization of the collagenous material.

Coming to the studies by Charles Birnberg, I believe that, on regarding his results, we must ask first whether any advantages have been gained by the use f Pitocin alone and, second, whether the entire procedure, which A. F. Lash f Chicago, Ill., has termed "jet propulsion," is meritorious.

From Chicago comes the report by Williams and McMahon,[9] who used itocin drip in four main groups of patients.

irst, for induction:	
Primips: Average first stage	Three and one quarter hours
High	Four hours, 52 min.
Low	One hour, 14 min.
Multips: Average first stage	One hour, 48 min.
High	Four hours, 20 min.
Low	Twenty-eight min.
econd, as an aid to labor already in progress:	
Primips: First stage	One and one half hours
Multips: Average first stage	One hour, 16 min.
High	One hour, 39 min.
Low	Ten min.
hird, primary inertia:	
First stage after Pitocin was started	
Average	One hour, 48 min.
High	Four hours, 24 min.
Low	Fifteen min.
ourth, secondary inertia:	
First stage after Pitocin was started	
Average	Forty-six min.
High	Three hours
Low	Ten min.

These results compare favorably with those presented here today.

It has always seemed to me that the cervix contributes very little to the nolding of the head, though from his original report Birnberg states that the eneficial effect on the cervix by Cervilaxin helps to prevent molding. I vonder also what is the basis for his statement that increased amounts of Cerilaxin relax the perineum.

The important question, however, is whether precipitate and tumultuous abor is good for the infant, leaving aside the possibility of cervical and perineal acerations.

Eastman[2] defines precipitate labor as one lasting under three hours, and in iscussing this problem points out that the danger of such short labors is an ncreased infant mortality due to: (1) tumultuous contractions with very hort intervals between, preventing proper oxygenation of fetal blood; (2) too apid transit of the baby through the bony pelvis, producing cerebral trauma; 3) unattended delivery, because of the too rapid labor, and lack of proper mmediate care for the baby; and (4) sudden compression of the occult prolapsed ords or cord about the neck, a potentiality that cannot be overlooked.

To be sure, Pitocin is a valuable drug but, at the same time, it is a dangerous ne if not used carefully and with good indications.

The situation is clarified by the long-range studies of babies who were the roducts of such precipitate labors. In a fifteen-year follow up of such infants, enaron and Tucker[1, 8] have shown conclusively that the highest percentage f mental retardation and the greatest physical and neurological impairment ccurred in the children of patients whose labor continued for less than three hours. Consideration for the infant dictates that one should be careful about propelling the infant's brain through the birth canal tumultuously.

Finally, we come to the problem of the use of relaxin in dysmenorrhea. Our preliminary results differ from those of Herbert S. Kupperman, possibly because we have used Releasin alone.

Time does not permit a full presentation or discussion of our results. Briefly, 5 different groups were given increasing dosage of oral Releasin starting with 4 mg. and increasing to 6, 8, 10, and 15 mg. every hour for 3 to 6 doses.

At a dosage of 15 mg. orally every hour, we experienced a failure in only 3 instances. Although the patient had very slight residual pain, this was insufficient to prevent her from resuming her work. A summary of all oral medication indicated a total failure in only 9.6 per cent. The most promising group is that comprising patients with intractable dysmenorrhea who did not respond to oral medication. Here we found the variation in time before complete relief after an intramuscular injection of 1 cc. of aqueous solution of Releasin to be 15 min. to 2½ hours, with an average of 56.8 min. Seventy-three per cent had complete relief in less than 1 hour.

We must conclude from all the studies thus far completed that relaxin is undoubtedly a factor to be dealt with in various aspects of the nonpregnant and pregnant uterus. Though there is still some skepticism as to its value in premature labor, I believe it should be used only in selected cases. I further believe it will prove to be of great value in conditioning the cervix prior to the induction of labor and that it holds great promise in the treatment of dysmenorrhea. If our present work corroborates our previous findings regarding its effectiveness as an oral medication, then its use as an adjunct to progesterone in the treatment of threatened and repeated abortions will be widely endorsed.

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B. G. Steinetz (*Warner-Lambert Reasearch Institute, Morris Plains, N. J.*) The potency of Releasin is expressed in milligram equivalents of Warner-Chilcott relaxin reference standard W1164-A, Lot 8. Each lot of Releasin is bioassayed before and after being processed into the sterile vialed solution.

An objective, quantitative bioassay method is employed. The technique is based on the dose-proportional increase observed in the length of interpubic ligaments of estrogen-primed mice given single injections of relaxin. Ligament lengths are measured precisely using transillumination and magnification of dissected pubic symphyses. For each assay, 120 or more mice are equally distributed among 3 or 4 dose levels for each of "standard" and "unknown." Limits of error of potency estimates are usually within −23 to +35 per cent at $p = 0.95$. The index of precision (λ) is generally 0.2 to 0.3.

Releasin was formerly standardized by a subjective but carefully controlled guinea pig pubic symphysis palpation assay by which the current reference standard has an activity of 150 guinea pig u./mg. For over two years Releasin lots were assayed by both methods against the current reference standard, and the potency estimates obtained by the 2 methods are in excellent agreement.

Although, classically, relaxin is defined by its action on the pubic symphysis, an *in vitro* assay based on uterine-motility inhibition in the estrous mouse has also been employed as a check on the "uterine-relaxing factor" activity of relaxin-containing extracts. Here again, "unknowns" are tested against the relaxin standard. Results obtained with this method are generally in good agreement with corresponding pubic symphysis assays.

A detailed comparison and a statistical evaluation of relaxin bioassay methods are included in the paper by Kroc *et al.* in this publication.

Harold H. Bryant (*Hynson, Westcott & Dunning, Inc., Baltimore, Md.*): The procedure used for biological assay and standardization of Lutrexin* is strictly an *in vivo* method. The test is designed to ascertain the amount of uterine-relaxing hormone present and is not a procedure suitable for assay of relaxin per se. While it is true that Lutrexin preparations do contain some relaxin, the Lutrexin assay procedure will not, and does not, serve as a suitable means of assay of the amount present.

Furthermore, the Lutrexin assay method is not a "uterine strip" or excised muscle procedure. The test involves the entire animal, as the uterus is completely intact with full nerve and blood supply. The material being assayed is injected intravenously or administered orally. In the routine assay, the test material is injected intravenously because of the sharp end point obtained following this route of administration. To anyone familiar with the problems of biological standardization, the desirability of a sharp end point is obvious. The accuracy of a bioassay is directly proportional to the sharpness of a pharmacological response being observed. However, the Lutrexin preparation is also administered orally, and the pharmacological response in decreased uterine activity is readily apparent. The dosage is, of course, higher, and the effect is not as quickly apparent, as is true of all pharmacological agents. The

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* Hynson, Westcott & Dunning, Inc., Baltimore, Md.

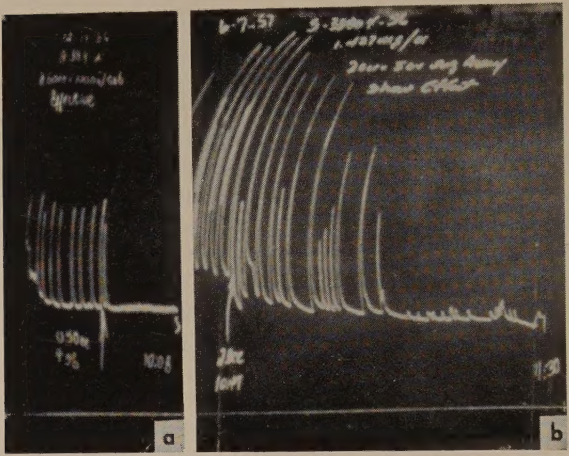

FIGURE 1. Photographs of assay tracings demonstrating the response to intravenous (a) and orally (b) administered Lutrexin. Note the sharp end point following intravenous injection. Also, observe the effective inhibition of uterine activity following oral administration.

dose required for clinical effectiveness has been determined by the physician administering the drug orally. The laboratory assay of the drug simply determines and standardizes the potency to enable the clinician to administer a "known" amount.

The uterine strip is of very limited value, especially with the water-soluble hormones of the ovary. The original publication (Krantz *et al.*, 1950) describing the assay procedure noted that 15 of 26 substances studied, using excised uterine strips, brought about a diminution of uterine activity. The uterine strip is then completely nonspecific and not suitable for assay purposes.

The *in vivo* relaxation of the intact uterus by the ovarian extract has proved to be quite specific. Roughly one hundred substances studied during the past ten years using the Lutrexin assay procedure have failed to give similar inhibition of uterine activity (FIGURE 1).

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WILLIAM KLEINBERG (*Princeton Laboratories, Princeton, N. J.*): The animal assay of Cervilaxin in terms of guinea pig units was determined by competent investigators and the product still conforms to the Abramowitz unit.[1]

On the basis of accumulating data indicating the quantitation of Cervilaxin in terms of human cervical softening, a human assay is proposed.

Human parturifacient action, the only claim presently made for Cervilaxin, is probably not related to activity on the guinea pig or mouse pubic symphysis.

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